## AMENDMENTS TO THE DRAWINGS

A replacement sheet is submitted herewith for Figure 14A1, A2, B1, B2. This is sheet 16/16 of the drawings. The replacement drawing is a lighter exposure and is printed on regular paper so that is will be legible after scanning.

## REMARKS

Claims 1-3 have been cancelled. Claims 4-8 and 12 have been amended. New claims 13-15 are added. Claims 4-15 are now pending in this application. Claims 7-11 are withdrawn. Claims 7 and 8 have been amended to depend from claim 4 as claim 1 has been cancelled. Applicants request rejoinder of claims 7-11 upon allowance of claim 4. Support for the amendments is found in the existing claims and the specification as discussed below. Accordingly, the amendments do not constitute the addition of new matter. Applicant respectfully requests the entry of the amendments and reconsideration of the application in view of the amendments and the following remarks.

**Drawings**

A replacement sheet is submitted herewith for sheet 16/16 (Figure 14 A1, A2, B1, B2), in response to the Examiner's objection that the originally submitted drawing was too dark. The replacement drawing is identical to the originally filed drawing but is a lighter exposure and is printed on regular paper so that it will be legible after scanning.

**Objections to the specification**

The specification has been amended to address the error noted by the Examiner that the references to SEQ ID NO: 2 are off by one residue in most instances. In addition, other clerical errors have been corrected including the spelling of "osteoporosis", and the MIN No. of the osteoporosis-pseudoglioma syndrome in Example 8. In paragraph 0120, the last "214 amino acids" has been corrected to "162 amino acids". However, it is noted that Plaisant et al. (2003) (Attachment A, discussed below) refers to "161 amino acids" (notably, the first methionine was excluded from the sequence, and consequently, the cysteine of paragraph 120 is located at position 47).

The continuing data section has been updated to show the patent number for the parent application.

**Claim objections**

Claim 5 has been amended to correct the numbering of amino acid positions which is off by one residue in all positions except for the leucine in position 162 which appears to be correct.

In view of Applicants' amendments to the specification and claims, reconsideration and withdrawal of the objections is respectfully requested.

**Rejection under 35 U.S.C. § 112, first paragraph**

Claims 1-6 and 12 are rejected under 35 U.S.C. § 112, first paragraph because the specification, while enabling for (1) a polypeptide comprising SEQ ID NO: 2 for treating ibotenate induced brain lesions, and (2) A peptide consisting of the amino acid sequence selected from the group consisting of the sequence between the glutamic acid in position 14 and the glutamic acid in position 28 of SEQ ID NO: 2, the sequence between the alanine in position 27 and the leucine in position 37 of SEQ ID NO: 2, the sequence between the alanine in position 43 and the glutamic acid in position 58 of SEQ ID NO: 2, the sequence between the glutamic acid in position 58 and the valine in position 70 of SEQ ID NO: 2, the sequence between the valine in position 81 and the leucine in position 98 of SEQ ID NO: 2, the sequence between the arginine in position 96 and the leucine in position 113 of SEQ ID NO: 2, the sequence between the serine in position 119 and the serine in position 130 of SEQ ID NO: 2, the sequence between the valine in position 138 and the threonine in position 151 of SEQ ID NO: 2, the sequence between the glutamic acid in position 14 and the cysteine in position 48 of SEQ ID NO: 2, the sequence between the glutamic acid in position 14 and the glycine in position 39 of SEQ ID NO: 2, the sequence between the leucine in position 37 and the cysteine in position 48 of SEQ ID NO: 2, and the sequence between the threonine in position 151 and the leucine in position 162 of SEQ ID NO: 2 for diagnosing osteoarthritis cartilage or making antibody, does not reasonable provide enablement for (1) any amino acid sequence having more than 70%, 85% or 95% homology with SEQ ID NO: 2, (2) any portion of SEQ ID NO: 2 as set forth in claim 5 previously, and (3)/(4) amino acid sequences, pharmaceutical formulations or pharmaceutical salts, or derivatives having more than 70% homology with SEQ ID NO: 2.

This ground of rejection is overcome in part by amendment and in part by argument.

With respect to claim 5, without acquiescing to the Examiner's position, claim 5 has been amended to the fragments indicated as enabled by the Examiner. Accordingly, this ground of rejection may be withdrawn for claim 5.

Regarding claims 1-4, claims 1-3 have been cancelled. This ground of rejection is moot for claims 1-3.

Claim 4 has been amended to independent form. Applicants' assert that the present specification is completely enabling for sequences having at least 95% homology to SEQ ID

NO:2. The application discloses not only SEQ ID NO: 2, which relates to the human PRDX5 sequence, but also SEQ ID NOS: 4 and 6, which relate to the rat and mouse PRDX5 sequence, respectively. Notably, the mitochondrial targeting pre-sequence is missing from these sequences. Upon aligning SEQ ID NOS: 2, 4 and 6, 12 amino acids differed over a total 162 amino acids. This reflects an overall homology of about 93% between the three sequences and also provides guidance to one skilled in the art as to which residues may be modified with retention of activity. See also Figure 5 of the present specification which shows homology between the human and rat B18 (PRDX5) amino acid sequences as 97.5% and between the human and mouse B18 (PRDX5) sequences as 96%. Accordingly, Applicants respectfully submit that the present specification is enabling for sequences having 95% homology as two additional sequences having more than 95% homology to SEQ ID NO: 2 are described in the present specification. Furthermore, an alignment of the three sequences provides sufficient guidance to one skilled in the art to determine which residues may be replaced or deleted without loss of biological activity.

Regarding utility, the Examiner admits that SEQ ID NO: 2 has utility for treating ibotenate induced brain lesions and that the subsequences of claim 5 have utility for diagnosing osteoarthritis cartilage or making antibody (see item 10 on page 4 of paper no. 03032006). Furthermore, with respect to the fragments as claimed in claim 5, support for the asserted utility is disclosed in the present specification at paragraphs 0041-0046 which discloses use of peptide fragments to generate antibodies and their use in a diagnostic device such as a kit or column.

Regarding the utility of SEQ ID NO: 2, (claims 4, 6, and 12-15), the specification discloses at paragraph 0134 that "intraperitoneal administration of recombinant PRDX5 immediately after ibotenate produced a dose-dependent protection against the ibotenate-induced cortical plate and white matter lesions (Figures 10B and 11A)". Also, as disclosed in paragraph 0137, "tunel staining performed at 8 and 48 hours following ibotenate injection showed that recombinant PRDX5 induced a significant reduction of cortical plate [and white matter cell death] (Figure 12)". As discussed in paragraph 0138, when compared to control, exposure to PRDX5 induced a reduction of NMDA-induced neuronal cell death (Figure 13A) and co-treatment with PRDX5 and DTT induced a larger reduction of NMDA-induced cell death.

In further support of the evidence provided by the present specification, Applicants' present two articles from peer reviewed journals which relate to the present application (see Attachment A).

Plaisant, et al. "Recombinant Peroxiredoxin 5 protects Against Excitotoxic Brain Lesions in Newborn Mice" (2003) Free Radical Biology & Medicine, vol. 34, no.7: pages 862-872.

Wang, et al. "Expression and Regulation of Peroxiredoxin 5 in Human Osteoarthritis" (2002) FEBS Letters vol. 531: pages 359-362.

The article by Plaisant, et al. describes data corresponding to the data presented in the present specification. The article by Wang , et al. provides evidence for a role for PRDX5 in osteoarthritis and concludes that "PRDX5...may have therapeutic value in the prevention and treatment of OA". Applicants would like to emphasize that the acceptance and publication of this data by peer reviewed journals underscores the scientific reliability and reproducibility of the present invention.

Accordingly, Applicants respectfully submit that SEQ ID NO: 2, per se, and all of the subsequences of claim 5 as amended have a utility.

Regarding sequences homologous to SEQ ID NO: 2, the Examiner takes the position that even small changes in sequence can result in loss of biological activity and that close sequence identity is no guarantee of biological activity. The Examiner cites Mason, et al. for their teaching that replacement of cysteine residues in activin A results in loss of activity. There is also a mention of TGFβ2 at page 330, col. 1. However, this reference does not relate to PRDX5 sequences. Mason, et al. is a specific teaching for activin A. At best, Mason, et al. may be pertinent to dimers linked by a disulfide bond. However, Mason, et al. is not pertinent here.

Atwood, et al. cited by the examiner, relates to bioinformatics and not to the present case where a function may be assigned to a specific sequence. The present claims relate to a specified structure characterized by SEQ ID NO: 2, which has an experimentally proven function, in contrast to the computationally predicted functions of possible proteins of Atwood. Nevertheless, Atwood admits that function prediction through pattern recognition is possible (page 2, penultimate paragraph), contrasting the opinion by the examiner on page 6, line 6 *et seq* stating

that "There is no recognition in the art that sequence identity [sic.] predicts biological function and therefore a disclosure of sequence identity does not lead one of skill in the art at the time the invention was made to believe said identity gives a credible use to the claimed protein." Accordingly, neither Mason, et al. nor Atwood, et al. support the Examiner's position with respect to the present case.

Furthermore, as discussed above, the present claims are now limited to sequences with at least 95% homology to SEQ ID NO: 2. Two sequences falling within the scope of this claim are described in the application, besides SEQ ID NO: 2, per se. These sequences are the corresponding sequences from rat (SEQ ID NO: 4) and mouse (SEQ ID NO: 6). These sequences are all identified as PRDX5 (B18). As disclosed in the application at paragraph 0021, the term "PRDX5" is used to denote an activity similar to the activity by the amino acid sequence of SEQ ID NO: 2". Accordingly, the mouse and rat sequences (SEQ ID NOS: 4 and 6) having greater than 95% homology with SEQ ID NO: 2, and designated as PRDX5 sequences have similar activity to SEQ ID NO: 2. As discussed above, alignment of these three sequences indicates 12 residues that are not identical. Accordingly, one skilled in the art would know that modification at these sites could be made with retention of biological activity.

On page 7, first sentence, the Examiner states that "it is known that autoimmune osteoarthritis is model dependent" and implies that intracerebral administration to a mouse pup is an inappropriate model for autoimmune osteoarthritis. However, this statement is made without any supporting evidence and so may be set aside. As set forth in a letter to the Examining Corps dated February 21, 2002 (see Attachment B),

> It would not be appropriate for the examiner to take official notice of facts without citing a prior art reference where the facts asserted to be well known are not capable of instant and unquestionable demonstration as being well-known. For example, assertions of technical facts in the areas of esoteric technology or specific knowledge of the prior art must always be supported by citation to some reference work recognized as standard in the pertinent art. (*In re Ahiert,* 424 F.2d at 1091, 165 USPQ at 420-21. See also *In re Grose, 592* F.2d 1161, 1167-68, 201 USPQ *57,* 63 (CCPA 1979) ("[w]hen the PTO seeks to rely upon a chemical theory, in establishing a prima facie case of obviousness, it must provide evidentiary support for the existence and meaning of that theory."); *In re Eynde,* 480 F.2d 1364, 1370, 178 USPQ 470, 474 (CCPA 1973) ("we reject the notion that judicial or administrative notice may be taken of the state of the art. The facts constituting the state of the art are normally subject to the possibility of rational disagreement among reasonable men and are not amenable to the taking of such notice.").

The Examiner states that the specification does not teach a possible reductant or electron donor which should be included in the claimed pharmaceutical formulation (Office Action, page 7, first paragraph). In response, without acquiescing to the Examiner's position, Applicants have deleted this language from claim 6. New claim 15 is added which specifically recites DTT which is supported in the specification at paragraph 0138.

Regarding claim 5, the Examiner states that the claim is not enabled for the open language as recited. In response, without acquiescing to the Examiner's position, Applicants have amended claim 5 to recite closed language, copying the language provided by the Examiner on page 4, item 10, lines 3-13, which the Examiner has indicated is enabled.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

**Rejection under 35 U.S.C. § 112, first paragraph**

Claims 1-6 and 12 are rejected under 35 U.S.C. § 112, first paragraph as containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s) had possession of the claimed invention at the time that the application was filed.

This ground of rejection has been addressed above and the comments made above are incorporated herein by reference. The claims are now limited to SEQ ID NO: 2, the specific subsequences listed in claim 5, and sequences which have 95% homology with SEQ ID NO: 2. SEQ ID NO: 2 and the specific subsequences of claim 5 are described in the present specification. Regarding support for sequences having 95% homology with SEQ ID NO: 2, such support is found in Figure 5, which shows SEQ ID NOS: 4 and 6 having at least 95% homology with SEQ ID NO: 2.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

**Rejection under 35 U.S.C. § 112, second paragraph**

Claim 6 is rejected under 35 U.S.C. § 112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The word "possibly" has been deleted from claim 6. Accordingly, this ground of rejection may be withdrawn.

**Rejection under 35 U.S.C. § 102(e)**

Claims 1-6 and 12 are rejected under 35 U.S.C. § 102(e) as being anticipated by US Patent No. 6,197,543.

Applicants note that this reference was also cited in the parent application, U.S. Application No. 09/486,167. As set forth in the response of December 18, 2002 in that application, the present application claims priority to Belgium application no. 9700692 (Attachment C), filed August 20, 1997, which predates the filing date of the '543 patent that was filed on October 28, 1997. SEQ ID NO: 2 is entitled to the August 20, 1997 priority date, and thus U.S. 6,197,543 cannot be considered as prior art.

Specifically, in Figure 5, Belgian application 9700692 discloses the amino acid sequence of SEQ ID NO: 2 (designated in the text also as SEQ ID NO: 1). On page 5, line 22 to page 6, line 4, Belgian application 9700692 refers to homologues of at least 70%, preferentially more than 85% and even more preferentially over 95% homology with the amino acid sequence of Figure 5. Moreover, Belgian application 9700692 relates to fragments thereof.

With respect to claim 5, this ground of rejection is also addressed by amendment. the '543 patent does not disclose the specific subsequences of claim 5.

In view of Applicants' amendments, arguments and resubmission of a copy of Belgium application no. 9700692, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

**CONCLUSION**

In view of Applicants' amendments to the claims and the foregoing Remarks, it is respectfully submitted that the present application is in condition for allowance. Should the Examiner have any remaining concerns which might prevent the prompt allowance of the application, the Examiner is respectfully invited to contact the undersigned at the telephone number appearing below.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: July 13, 2006

By: _____

Che Swyden Chereskin, Ph.D.
Registration No. 41,466
Agent of Record
Customer No. 20,995
(949) 721-6385

2738264
071006

# ATTACHMENT A

FEBS 26677 FEBS Letters 531 (2002) 359–362

# Expression and regulation of peroxiredoxin 5 in human osteoarthritis

Min-Xia Wang[a], Aiqun Wei[a], Jun Yuan[a], Annette Trickett[b], Bernard Knoops[c], George A.C. Murrell[a,*]

[a] *Orthopaedic Research Institute, St George Hospital, University of New South Wales, Sydney, NSW 2217, Australia*
[b] *Clinical Hematology, St George Hospital, University of New South Wales, Sydney, NSW 2217, Australia*
[c] *Laboratory of Cell Biology, ISV, Université Catholique de Louvain, Place Croix du Sud, 5, 1348 Louvain-la-Neuve, Belgium*

Received 10 July 2002; revised 30 August 2002; accepted 4 September 2002

First published online 16 October 2002

Edited by Veli-Pekka Lehto

**Abstract Reactive oxygen species (ROS) are implicated in the pathogenesis of osteoarthritis (OA). However, little is known about the antioxidant defence system in articular cartilage. We investigated the expression and regulation of peroxiredoxin 5 (PRDX5), a newly discovered thioredoxin peroxidase, in human normal and osteoarthritic cartilage. Our results show that human cartilage constitutively expresses PRDX5. Moreover, the expression is up-regulated in OA. Inflammatory cytokines tumour necrosis factor α and interleukin 1 β contribute to this up-regulation by increasing intracellular ROS production. The present study suggests that PRDX5 may play a protective role against oxidative stress in human cartilage.**


*Key words:* Osteoarthritis; Peroxiredoxin 5; Oxidative stress; Reactive oxygen species; Articular cartilage; Antioxidant

## 1. Introduction

Osteoarthritis (OA) is the most common form of arthritis and is characterised by chronic pain and significant disability. Although the aetiology of OA is multifactorial, the key pathological feature of OA is articular cartilage degradation [1]. The pathological processes of cartilage degradation, including the molecular processes that impair cartilage homeostasis, are still largely unknown.

Several studies have demonstrated that chondrocytes are able to generate reactive oxygen species (ROS) under certain conditions [2–4]. The over-production of ROS molecules including superoxide ($O_2^{\bullet-}$), hydrogen peroxide ($H_2O_2$), hydroxyl radicals ($^{\bullet}OH$) and nitric oxide ($NO^{\bullet}$) may contribute to the degradation of cartilage matrix. ROS can inhibit proteoglycan and hyaluronic acid syntheses [5], fragment hyaluronan [6] and enhance matrix protein degradation [7]. ROS also damage cell membranes [8] and induce apoptosis of synoviocytes [9]. Although there is information regarding the pathological role of ROS in cartilage degeneration, little is known about the antioxidant defence system and particularly about any ROS scavengers in articular cartilage. The redox status of human chondrocytes in OA is also largely undetermined.

To prevent toxicity by ROS, mammalian cells possess a well-coordinated antioxidant enzyme system. Examples of antioxidant enzymes that have been well characterised are the superoxide dismutases (SOD) which catalyse superoxide to $H_2O_2$ [10]. SOD play a critical but limited role in detoxifying ROS, as $H_2O_2$ can cause oxidative stress if the levels of the $H_2O_2$ detoxification enzymes are insufficient or depleted. Catalase and glutathione peroxidases are widely investigated $H_2O_2$-scavenging enzymes. Recently, a novel family of peroxidases, the peroxiredoxins (PRDX), was identified in many living organisms. Six isoforms of PRDX have been identified in mammals [11–13], all of which participate directly in eliminating $H_2O_2$ and neutralising other oxidising chemicals [14]. A study on the crystal structure of human peroxiredoxin 5 (PRDX5), also known as PrxV/AOEB166/PMP20/ARC1 [12,15–17], suggested that PRDX5 may have a broader activity against ROS compared with other isoforms of PRDX and other antioxidant enzymes [18]. We have previously shown that the expression of PRDX5 is up-regulated in degenerative human tendon [19]. The expression of this enzyme in human cartilage, particularly in osteoarthritic cartilage, has not been investigated.

To understand better the mechanisms by which human chondrocytes are protected against oxidative stress, we investigated the expression of PRDX5 in normal and osteoarthritic human cartilage, and investigated the regulation of PRDX5 expression in human chondrocytes. We were particularly interested in determining if inflammatory cytokines play any role in the regulation of PRDX5 expression.

## 2. Materials and methods

### 2.1. Tissue collection and culture

Human tissue collection in this study was approved by the South Eastern Sydney Area Health Service Ethics Committee, Australia. Human osteoarthritic cartilage ($n=4$) was removed from knee joints of patients with OA undergoing total knee-replacement surgery. Significant OA was identified by preoperative radiography. Normal human cartilage ($n=4$) was removed immediately adjacent to the insertion site of supraspinatus tendon as part of the surgical procedure to reattach the tendon to bone. Significant OA was excluded by plain anterior–posterior and lateral radiographs of the shoulder, and by arthroscopic examination of the glenohumeral joint prior to open rotator cuff repair.

For RNA and protein extraction, cartilage tissues were snap-frozen in liquid nitrogen upon collection and stored at −80°C until RNA extraction and tissue homogenisation were performed. For tissue culture, cartilage explant discs were made using a 4-mm-diameter disposable biopsy punch (SMS Inc., Columbia, MD, USA) with an average thickness of 2 mm. Three discs were placed in each well of 24-well

*Corresponding author. Fax: (61)-2-93503967.
*E-mail address:* admin@ori.org.au (G.A.C. Murrell).

*Abbreviations:* OA, osteoarthritis; PRDX5, peroxiredoxin 5; ROS, reactive oxygen species

0014-5793/02/$22.00 
PII: S0014-5793(02)03511-1

plates (1 ml final volume). Chondrocytes were isolated from cartilage by collagenase digestion [20] and plated in 25-cm$^2$ flasks ($5\times10^5$) containing Dulbecco's modified essential medium (DMEM) supplemented with 10% foetal bovine serum. Primary chondrocyte monolayer cultures and cartilage explants were incubated at 37°C in a 5% $CO_2$ humidified atmosphere. Culture medium was replaced with serum-free DMEM for all experiments.

To mimic in vivo pathological conditions, tumour necrosis factor α (TNFα) 100 ng/ml, and interleukin 1 β (IL-1β) 10 ng/ml (R&D System, Minneapolis, MN, USA) were added to explant and chondrocyte cultures. Catalase (500 U/ml) (Sigma, St. Louis, MO, USA) was used as an $H_2O_2$ scavenger and added to chondrocyte culture 2 h before TNFα stimulation. Tissue and cells were harvested at 3, 6, 12, 24 and 48 h after the addition of TNFα or IL-1β for total RNA extraction, protein preparation and intracellular $H_2O_2$ determination.

*2.2. Northern blotting*

Total RNA was isolated from human chondrocytes using Trizol reagent (Life Technologies, Melbourne, Australia) following the manufacturer's instructions. Denatured RNA samples (20 μg) were fractionated by electrophoresis in a denaturing 1% (w/v) agarose gel, transferred to a GeneScreen Plus nylon membrane (NEN Life Science Products, Boston, MA, USA), cross-linked using an ultraviolet cross-linker (Ultra-Lum, Carson, CA, USA), and hybridised with $^{32}$P-labelled human PRDX5 cDNA probe. The blots were subsequently stripped and reprobed with $^{32}$P-labelled human β-actin cDNA. All probes were radiolabelled by random priming (Promega, Sydney, Australia).

*2.3. Western blotting*

Human cartilage tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle, and homogenised in five volumes of Tris–HCl buffer (pH 7.4) containing a protease inhibitor cocktail (2 μg/ml leupeptin, 5 μg/ml pepstatin A, 2 mg/ml of ethylenediamine tetraacetic acid (EDTA)–$Na_2$, 500 μg/ml AEBSF and 1 μg/ml E-64) (Sigma). Chondrocyte lysate was prepared by sonication in the same homogenisation buffer. Proteins in the tissue homogenates or cell lysate were denatured by boiling for 5 min in 2% (w/v) sodium dodecyl sulfate (SDS) sample buffer and fractionated by electrophoresis on 12.5% (w/v) SDS–polyacrylamide gel. The fractionated proteins were electrophoretically transferred to a polyvinylidene difluoride membrane, and the blots were blocked with 5% (w/v) non-fat dry milk TTBS solution containing 25 mM Tris, pH 7.5, 150 mM NaCl, 0.05% (w/v) Tween 20. After washing in TTBS, the blots were incubated for 1 h with polyclonal anti-PRDX5 antibody [19] at 1:2000 dilution, followed by horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Chemicon, Melbourne, Australia). Immunoreactive bands were detected by enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA). The membranes were stripped with Restore Western Blot Stripping Buffer (Pierce) and reprobed using a rabbit anti-human actin antibody (Sigma) as a house-keeping control. The net intensity of the PRDX5 band was analysed by Bio-Rad Quantity One image quantification system (Bio-Rad, Hercules, CA, USA). PRDX5 protein level was expressed by the ratio of the net intensity of the PRDX5 band to actin band of the same sample.

*2.4. Intracellular $H_2O_2$ assay*

Intracellular $H_2O_2$ generation was assessed in chondrocytes by a flow cytometric technique based on the methods of Sattler et al. [21], in which 2′,7′-dichloro-fluorescin-diacetate (DCF-DA) (Sigma) was used. DCF-DA is a cell-permeable dye commonly used to monitor intracellular changes in ROS (more specifically for $H_2O_2$). DCF-DA readily diffuses into cells where it is hydrolyzed to the non-fluorescent derivative 2′,7′-dichloro-fluorescin (DCF) and is trapped within the cells. DCF becomes highly fluorescent when oxidised by either $H_2O_2$ or superoxide. Cellular fluorescence intensity is directly proportional to the level of intracellular $H_2O_2$ produced by the cells and can be monitored by flow cytometry. Chondrocytes were freed from culture flasks by trypsin–EDTA. Cells ($10^6$) were incubated with 5 μM of DCF-DA for 5 min at 37°C and subsequently washed twice in cold phosphate-buffered saline before analysis by a flow cytometer (FACSort, Becton Dickinson System, San Jose, CA, USA) with Cell Quest Software (Becton Dickinson). The fluorescence of oxidised DCF was measured with an excitation wavelength of 488 nm and emission wavelength of 525 nm. Mean fluorescence intensity (MFI) for 10 000 cells was recorded for each sample. Fluorescence relative variance (FRV) was calculated for each sample (S) compared with control (C; loaded cells without TNFα treatment): $FRV=[(MFI_{(S)}-MFI_{(C)})/MFI_{(C)}$.

*2.5. Statistical analysis*

Comparison of PRDX5 expression in normal and osteoarthritic cartilage was made by Mann–Whitney Rank Sum Test using SigmaStat (Jandel Scientific, San Rafael, CA, USA). A $P$ value $<0.05$ was considered statistically significant.

## 3. Results

*3.1. PRDX5 protein is constitutively expressed in human cartilage and up-regulated in osteoarthritic cartilage*

A rabbit antibody was raised against recombinant human PRDX5 [19]. With this antibody, PRDX5 protein expression was readily detected by Western blot of cartilage tissue homogenates (Fig. 1). To semi-quantify the PRDX5 protein expression in both normal and osteoarthritic cartilage, all blots were reprobed with an anti-actin antibody and the results were expressed as a density ratio of PRDX5:actin. It was observed that osteoarthritic cartilage had more fibrin tissue and less cellularity than normal cartilage. Even if the same amount of total protein were loaded for Western blotting, one would expect less cellular protein in osteoarthritic cartilage than in normal cartilage. Therefore, the normalisation of PRDX5 protein with actin is important for the semi-quantification. Fig. 1 shows a significantly higher PRDX5 protein level in osteoarthritic cartilage than in normal cartilage, suggesting an up-regulation of PRDX5 in OA.

*3.2. PRDX5 expression is regulated by inflammatory cytokines*

To explore the mechanisms by which the PRDX5 expression in human chondrocytes is regulated, we introduce inflammatory cytokines to the cartilage explant culture and primary chondrocyte culture, as cytokines have been shown to be implicated in OA. As shown in Fig. 2, TNFα and IL-1β up-regulated PRDX5 protein expression in the cartilage explant

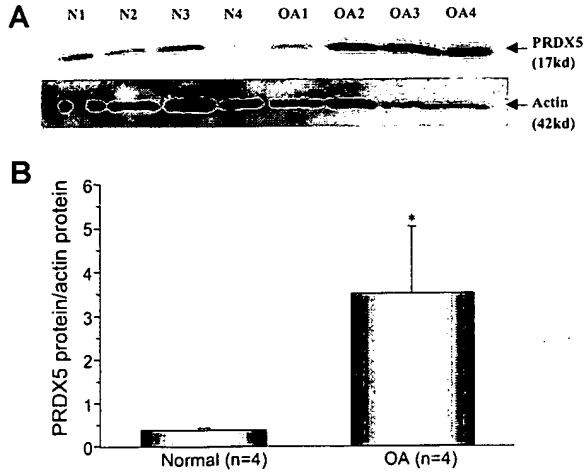


Fig. 1. Expression of PRDX5 protein in human articular cartilage. A: PRDX5 protein bands (upper panel) and actin bands (lower panel) detected by Western blotting in normal (N) and osteoarthritic cartilage (OA). B: Comparison of PRDX5 protein levels in normal and osteoarthritic cartilage ($n=4$) by densitometry analysis. PRDX5 protein level was expressed by the ratio of the net intensity of the PRDX5 band to the actin band of the same sample. Bars represent mean ± S.E.M. *$P<0.05$ OA vs normal.

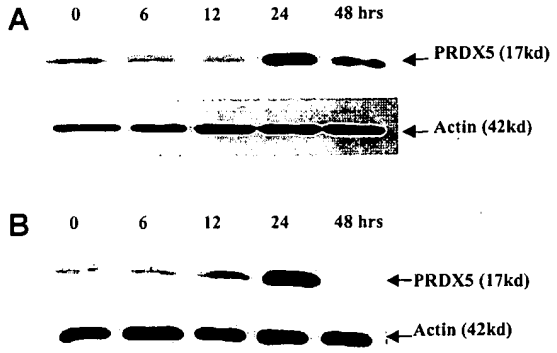


Fig. 2. Effects of TNFα and IL-1β on PRDX5 protein expression in human articular cartilage explant culture. Cartilage explants from OA patients were precultured with TNFα (100 ng/ml; panel A) or IL-1β (10 ng/ml; panel B) for the indicated times, and subject to Western blot analysis using anti-PRDX5 and anti-actin antibodies as described in Section 2.3.

culture 24 h after the cytokine challenge. At 48 h, the PRDX5 protein levels decreased to near baseline in TNFα-treated cartilage and below baseline in IL-1β-treated cartilage. A similar phenomenon was observed in primary chondrocyte culture (Fig. 3). Both PRDX5 mRNA and protein expression started to increase 3 h after TNFα stimulation, and reached their peak levels at 12 and 24 h. The protein level returned to baseline at 48 h, while the mRNA level remained high.

### 3.3. $H_2O_2$ might be an important mediator for the PRDX5 up-regulation in human chondrocytes

Cytokines can induce intracellular $H_2O_2$ production in chondrocytes. We hypothesised that the increased PRDX5 expression in stimulated chondrocytes was a cellular response to intracellular flux of $H_2O_2$. To test this hypothesis, intracellular $H_2O_2$ induction was measured using a flow cytometric technique. Catalase was introduced to the chondrocyte culture system to test if scavenging $H_2O_2$ would affect the PRDX5 expression level. As shown in Fig. 4A, the intracellular $H_2O_2$ level in cultured human chondrocytes started to rise 3 h after a TNFα challenge, reached its peak at 6 h, and started to decline at 12 h when the PRDX5 protein expression reached its peak (Fig. 4B). The addition of catalase to the chondrocyte

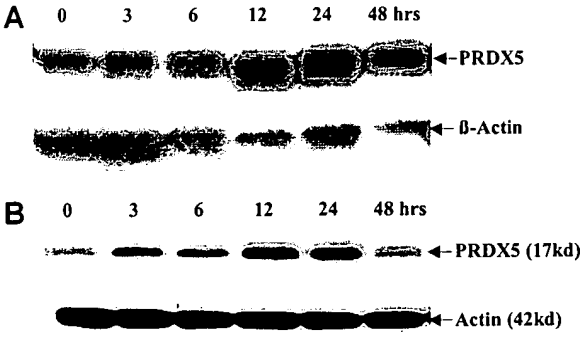


Fig. 3. Effects of TNFα on PRDX5 mRNA and protein expression in cultured human articular cartilage chondrocytes. Chondrocytes isolated from OA cartilage were precultured with TNFα (100 ng/ml) for the indicated times. A: PRDX5 mRNA was detected by Northern blot analysis. B: PRDX5 protein expression was analysed by Western blot.

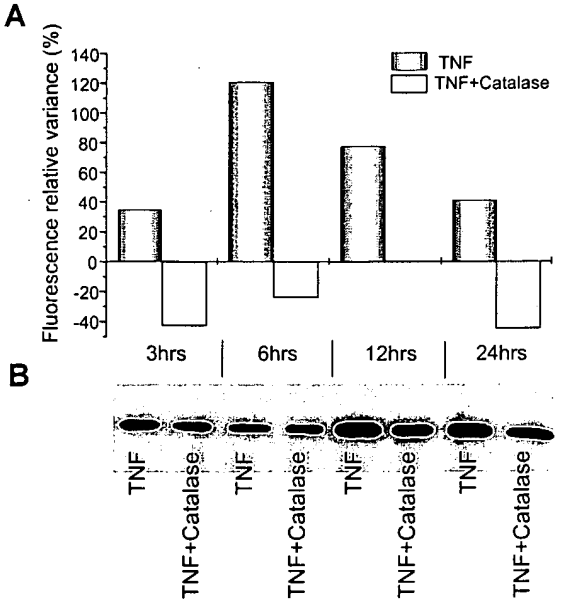


Fig. 4. Effect of catalase and/or TNFα on chondrocyte $H_2O_2$ production and PRDX5 protein expression. A: Flow cytometric analysis of intracellular $H_2O_2$ production by chondrocytes treated with TNFα (100 ng/ml) or TNFα+catalase (500 U/ml) for the indicated times. Catalase was added to culture 2 h before the addition of TNFα. B: Western blot analysis of PRDX5 protein from chondrocytes treated as above. Results were representatives of three separate experiments using chondrocytes isolated from three OA cartilages.

culture prior to exposure to TNFα significantly inhibited TNFα-induced intracellular $H_2O_2$ production (Fig. 4A), as well as the enhanced PRDX5 protein expression (Fig. 4B, at 12 and 24 h), suggesting that $H_2O_2$ might be an important mediator for the cytokine-induced PRDX5 up-regulation in human chondrocytes.

## 4. Discussion

Articular chondrocytes, embedded in an avascular matrix, are exposed to a low partial oxygen pressure and exhibit a predominantly anaerobic metabolism [22]. Accordingly, the ROS defence mechanisms in articular chondrocytes are generally weak and chondrocytes are susceptible to attacks by ROS. However, chondrocytes have the potential to increase their antioxidant status. Previous studies on chondrocytes antioxidant mechanisms have shown that human chondrocytes constitutively express SOD and catalase [23]. Our study provides the first evidence that human chondrocytes also express PRDX5 and the expression is significantly higher in osteoarthritic cartilage compared with normal cartilage.

To explore the regulatory mechanisms of the PRDX5 expression, we introduced the inflammatory cytokines TNFα and IL-1β to cartilage explant cultures and primary chondrocyte cultures, as these cytokines have been shown to stimulate chondrocyte ROS production [3,24] and to be implicated in OA [25]. Our results demonstrate that IL-1β and TNFα are important modulators of PRDX5 up-regulation. During the time course observation of PRDX5 expression, we noted a discrepancy between the PRDX5 mRNA and the protein levels in TNFα challenged chondrocytes. Indeed, the PRDX5 protein level returned to baseline 48 h after TNFα stimula-

tion, while the mRNA level remained high (Fig. 3). This may be due to a translational block for the synthesis of PRDX5 protein, which may have happened 48 h after initial PRDX5 up-regulation as a feed-back protection mechanism, as physiological levels of ROS are essential for many biochemical processes, including signal transduction [26], cell differentiation and immunity [27]. Translational regulation has been reported in many mammalian cells and is believed to be responsible for the discrepancy between mRNA and protein levels [28,29]. More experiments are necessary to define the translational regulation PRDX5 protein in human chondrocytes.

The pathways by which PRDX5 is up-regulated in OA are not yet known. It is possible that the up-regulation of PRDX5 is a response to increased levels of ROS in OA, as there is increasing evidence showing that over-production of ROS is implicated in the pathogenesis of OA [4,7,30,31]. Our study has shown that a TNFα/IL-1β challenge significantly increases intracellular $H_2O_2$ production, followed by enhanced PRDX5 expression. When the $H_2O_2$ scavenger catalase was introduced into the primary chondrocyte culture, intracellular $H_2O_2$ was inhibited and this was accompanied by the inhibition of PRDX5 protein expression, suggesting that $H_2O_2$ might be an important mediator for the cytokine-induced PRDX5 up-regulation.

The significantly increased expression of PRDX5 in OA may act as a protection to cartilage against ROS-induced oxidative damage. Increased SOD activity has been reported in OA [32]. Dismutation of the anion superoxide to $H_2O_2$ by SOD is a well-described defence mechanism against oxidative stress at the cellular level. However, reduction of $H_2O_2$ by the Fenton reaction to the highly toxic hydroxyl radical can be, in some circumstances, even more harmful to the cell. $H_2O_2$ detoxifying enzymes, including PRDX5, can metabolise and eliminate $H_2O_2$, partially avoiding the conversion of $H_2O_2$ to hydroxyl radical. Among the $H_2O_2$ detoxifying enzymes, catalase is known to be present only in peroxisomes [33]; glutathione peroxidase is restricted to the mitochondrial intermembrane space in very limited amounts [34]; PRDX5, however, is present in a broader range of intracellular locations, including mitochondria, peroxisomes and cytosol [12]. The functional significance of PRDX5 localisation in organelles needs further investigation.

In conclusion, our study has demonstrated that the antioxidant enzyme PRDX5 is constitutively expressed in human chondrocytes. The expression is up-regulated in OA. Inflammatory cytokines TNFα and IL-1β may be responsible for this up-regulation via the stimulation of intracellular $H_2O_2$ production. PRDX5 may play a protective role against oxidative stress involved in the pathogenesis of OA, and may have therapeutic value in the prevention and treatment of OA.

*Acknowledgements:* This study was supported by South Eastern Sydney Area Health Service and Health Care Australia/St. George Private Hospital. We thank Dr Allen Turnbull, Dr Robert Drummond and their patients for their generous support in human cartilage collection.

## References

[1] Martel-Pelletier, J. (1999) Osteoarthritis Cartilage 7, 371–373.
[2] Lo, Y.Y. and Cruz, T.F. (1995) J. Biol. Chem. 270, 11727–11730.
[3] Tiku, M.L., Liesch, J.B. and Robertson, F.M. (1990) J. Immunol. 145, 690–696.
[4] Tiku, M.L., Shah, R. and Allison, G.T. (2000) J. Biol. Chem. 275, 20069–20076.
[5] Bates, E.J., Lowther, D.A. and Johnson, C.C. (1985) Biochem. Biophys. Res. Commun. 132, 714–720.
[6] Panasyuk, A., Frati, E., Ribault, D. and Mitrovic, D. (1994) Free Radic. Biol. Med. 16, 157–167.
[7] Tiku, M.L., Gupta, S. and Deshmukh, D.R. (1999) Free Radic. Res. 30, 395–405.
[8] Fragonas, E., Pollesello, P., Mlinarik, V., Toffanin, R., Grando, C., Godeas, C. and Vittur, F. (1998) Biochim. Biophys. Acta 1425, 103–111.
[9] Galleron, S., Borderie, D., Ponteziere, C., Lemarechal, H., Jambou, M., Roch-Arveiller, M., Ekindjian, O.G. and Cals, M.J. (1999) Cell Biol. Int. 23, 637–642.
[10] Mazzetti, I., Grigolo, B., Pulsatelli, L., Dolzani, P., Silvestri, T., Roseti, L., Meliconi, R. and Facchini, A. (2001) Clin. Sci. (Lond.) 101, 593–599.
[11] Chae, H.Z., Kang, S.W. and Rhee, S.G. (1999) Methods Enzymol. 300, 219–226.
[12] Knoops, B., Clippe, A., Bogard, C., Arsalane, K., Wattiez, R., Hermans, C., Duconseille, E., Falmagne, P. and Bernard, A. (1999) J. Biol. Chem. 274, 30451–30458.
[13] Oberley, T.D., Verwiebe, E., Zhong, W., Kang, S.W. and Rhee, S.G. (2001) Free Radic. Biol. Med. 30, 412–424.
[14] Rhee, S.G., Kang, S.W., Netto, L.E., Seo, M.S. and Stadtman, E.R. (1999) Biofactors 10, 207–209.
[15] Seo, M.S., Kang, S.W., Kim, K., Baines, I.C., Lee, T.H. and Rhee, S.G. (2000) J. Biol. Chem. 275, 20346–20354.
[16] Kropotov, A., Sedova, V., Ivanov, V., Sazeeva, N., Tomilin, A., Krutilina, R., Oei, S.L., Griesenbeck, J., Buchlow, G. and Tomilin, N. (1999) Eur. J. Biochem. 260, 336–346.
[17] Yamashita, H., Avraham, S., Jiang, S., London, R., Van Veldhoven, P.P., Subramani, S., Rogers, R.A. and Avraham, H. (1999) J. Biol. Chem. 274, 29897–29904.
[18] Declercq, J.P., Evrard, C., Clippe, A., Stricht, D.V., Bernard, A. and Knoops, B. (2001) J. Mol. Biol. 311, 751–759.
[19] Wang, M.X., Wei, A., Yuan, J., Clippe, A., Bernard, A., Knoops, B. and Murrell, G.A. (2001) Biochem. Biophys. Res. Commun. 284, 667–673.
[20] Jang, D., Williams, R.J., Wang, M.X., Wei, A.Q. and Murrell, G.A. (1999) Arthritis Rheum. 42, 2410–2417.
[21] Sattler, M., Winkler, T., Verma, S., Byrne, C.H., Shrikhande, G., Salgia, R. and Griffin, J.D. (1999) Blood 93, 2928–2935.
[22] Stockwell, R. (1983) in: Cartilage (Hall, B., Ed.), Vol. 1, pp. 253–273, Academic Press, Orlando, FL.
[23] Deahl 2nd, S.T., Oberley, L.W., Oberley, T.D. and Elwell, J.H. (1992) J. Bone Miner. Res. 7, 187–198.
[24] Lo, Y.Y., Conquer, J.A., Grinstein, S. and Cruz, T.F. (1998) J. Cell. Biochem. 69, 19–29.
[25] Hedbom, E. and Hauselmann, H.J. (2002) Cell. Mol. Life Sci. 59, 45–53.
[26] Finkel, T. (1998) Curr. Opin. Cell Biol. 10, 248–253.
[27] Mates, J.M., Perez-Gomez, C. and Nunez de Castro, I. (1999) Clin. Biochem. 32, 595–603.
[28] Garcia-Sanz, J.A., Mikulits, W., Livingstone, A., Lefkovits, I. and Mullner, E.W. (1998) FASEB J. 12, 299–306.
[29] Pradet-Balade, B., Boulme, F., Beug, H., Mullner, E.W. and Garcia-Sanz, J.A. (2001) Trends Biochem. Sci. 26, 225–229.
[30] Murrell, G.A., Jang, D. and Williams, R.J. (1995) Biochem. Biophys. Res. Commun. 206, 15–21.
[31] Kurz, B., Jost, B. and Schunke, M. (2002) Osteoarthritis Cartilage 10, 119–126.
[32] Sumii, H., Inoue, H., Onoue, J., Mori, A., Oda, T. and Tsubokura, T. (1996) Hiroshima J. Med. Sci. 45, 51–55.
[33] Rabilloud, T., Heller, M., Gasnier, F., Luche, S., Rey, C., Aebersold, R., Benahmed, M., Louisot, P. and Lunardi, J. (2002) J. Biol. Chem. 277, 19396–19401.
[34] Rabilloud, T., Heller, M., Rigobello, M.P., Bindoli, A., Aebersold, R. and Lunardi, J. (2001) Proteomics 1, 1105–1110.

Free Radical Biology & Medicine, Vol. 34, No. 7, pp. 862–872, 2003

0891-5849/03/$–see front matter

ELSEVIER

doi:10.1016/S0891-5849(02)01440-5

*Original contribution*

# RECOMBINANT PEROXIREDOXIN 5 PROTECTS AGAINST EXCITOTOXIC BRAIN LESIONS IN NEWBORN MICE

FRANK PLAISANT,* ANDRÉ CLIPPE,† DELPHINE VANDER STRICHT,† BERNARD KNOOPS,† and PIERRE GRESSENS*

*INSERM E 9935 and Service de Neurologie Pédiatrique, Hôpital Robert-Debré, Paris, France; and †Laboratoire de Biologie Cellulaire, ISV, Université Catholique de Louvain, Louvain-la-Neuve, Belgium

(*Received* 17 *September* 2002; *Revised* 18 *November* 2002; *Accepted* 23 *December* 2002)

**Abstract**—The pathophysiology of brain lesions associated with cerebral palsy is multifactorial and likely involves excess release of glutamate and excess production of free radicals, among other factors. Theoretically, antioxidants could limit the severity of these brain lesions. Peroxiredoxins are a family of peroxidases widely distributed in eukaryotes and prokaryotes. Peroxiredoxin 5 (PRDX5) is a recently discovered mammalian member of this family of antioxidant enzymes that is able to reduce hydrogen peroxide and alkyl hydroperoxides. The present study was designed to examine the neuroprotective effects of recombinant PRDX5 against neonatal excitotoxic challenge in both in vivo and in vitro experiments. For in vivo experiments, mice (postnatal day 5) were injected intraneopallially with ibotenate acting on NMDA and metabotropic receptors, or S-bromowillardiine acting on AMPA-kainate receptors to produce excitotoxic stress and brain lesions. Systemically administered recombinant PRDX5 provided protection against ibotenate-induced excitotoxic stress. Brain lesions of animals given ibotenate and PRDX5 were up to 63% smaller than that given ibotenate alone. However, PRDX5 provided no prevention from lesions induced with S-bromowillardiine. A mutated recombinant PRDX5 that is devoid of peroxidase activity was also tested and showed no protection against lesions induced by either ibotenate or S-bromowillardiine. Two classical antioxidants, N-acetylcysteine and catalase-PEG, provided the same neuroprotective effect as PRDX5. For in vitro experiments, neocortical neurons were exposed to 300 $\mu$M NMDA alone, NMDA plus recombinant PRDX5, or NMDA, recombinant PRDX5 and dithiothreitol, a classical electron donor for peroxiredoxins. Recombinant PRDX5 plus dithiothreitol displayed a synergistic neuroprotective effect on NMDA-induced neuronal death. These findings indicate that reactive oxygen species production participates in the formation of NMDA receptor-mediated brain lesions in newborn mice and that antioxidant compounds, such as PRDX5, provide some neuroprotection in these models. 

**Keywords**—Peroxiredoxin, Catalase, Cerebral palsy, Glutamate, Neuronal cell death, N-acetylcysteine, Neuroprotection, *N*-methyl-D-aspartate, Free radicals

## INTRODUCTION

Injury to the perinatal brain is a leading cause of death and disability in children. While survival of very preterm infants has improved in the last decades, neurological handicap of perinatal origin (cerebral palsy) has not decreased in Western countries and may be increasing in some countries [1]. At the present time, there are no therapeutic regimens that can successfully treat injury to the developing brain. Much-needed research is required to assist affected children with reduced lifetime potential, families with emotional and financial burdens, and societies with staggering demands for resources to care for these children.

Periventricular leukomalacia (PVL) is the largest cause of cerebral palsy in preterm infants [1,2]. PVL consists of focal necrosis of periventricular white matter, which is often cystic. Recent studies have shown that PVL is often associated with gray matter abnormalities, including reduced cortical and hippocampal volume [3,4] and abnormal neuronal circuits [5]. Full-term human neonates with perinatal encephalopathy generally develop gray matter damage that most frequently affects the neocortex, the basal ganglia, and the hippocampus [6].

In recent years, the causes of CNS injury in human neonates have been considered to be multifactorial [7,8]. Many preconceptional, prenatal, and perinatal factors are

Address correspondence to: Dr. Pierre Gressens, Service de Neurologie Pédiatrique, Hôpital Robert-Debré, 48 Blvd Sérurier, 75019 Paris, France; Tel: +33(1)40-03-47-83; Fax: +33(1)40-03-47-74; E-Mail: gressens@idf.inserm.fr.

thought to cause injury to the developing brain, such as hypoxic-ischemic insults, endocrine imbalances, genetic factors, growth-factor deficiency, abnormal competition for growth factors, maternal infection yielding excess cytokines, and exposure to other proinflammatory agents. Several of these risk factors may share the same molecular pathways, such as excess release of excitatory amino acids and excess reactive oxygen species (ROS) production [9–12].

Research has shown that intracerebral injection of glutamate analogs in newborn rodents produces striatal, cortical plate, and cystic periventricular white matter lesions that mimic those observed in human neonates [13–20]. Excess activation of glutamate channel receptors on neurons induces a massive calcium influx, which leads to complex cellular changes. This is referred to as the excitotoxic cascade and includes an excessive production of ROS [21]. Increased production of ROS is also observed during the reperfusion phase, which follows a transient hypoxic-ischemic episode [22–25]. ROS causes cellular and tissue injury by damaging biologic macromolecules, including proteins, DNA, and lipids. Implication of ROS and reactive nitrogen species (RNS) in models of neonatal excitotoxic brain lesions is supported by the neuroprotective effects of inhibitors of nitric oxide synthase and 8-alkylamino-1,4-benzoxazine antioxidants [26,27].

Peroxiredoxins constitute a novel family of peroxidases whose members were identified in several mammalian species [28,29]. PRDX5, which is also called AOEB166/PrxV/PMP20/ACR1, is a recently cloned member of this family [30–34]. PRDX5 may be targeted intracellularly to mitochondria, peroxisomes, and the cytosol. The protein was initially isolated from human bronchoalveolar lavage fluid [35]. It is widely expressed in various tissues constitutively and upregulated during inflammatory processes [30,36]. PRDX5 exhibits peroxidase activity [30]. The antioxidant properties of PRDX5 are associated with peroxide reduction [30]. To our knowledge, neuroprotective properties of administered peroxiredoxins have not been evaluated to date.

The goal of the present study is to assess the neuroprotective effects of PRDX5 using a well-defined mouse model of neonatal excitotoxic brain lesions, which mimics several aspects of brain damage associated with human cerebral palsy.

## MATERIALS AND METHODS

### *Animals and drugs*

Swiss mice of both sexes were used for this study. All experimental protocols and procedures, were approved by the Institut National de la Santé et de la Recherche Médicale (INSERM). Ibotenate (Tocris, Bristol, UK) was diluted in phosphate buffer saline (PBS) containing 0.01% acetic acid. S-bromowillardiine (Tocris), recombinant human PRDX5, mutated recombinant human PRDX5, catalase (Sigma, Saint Quentin Fallavier, France), catalase-polyethylene glycol (catalase-PEG; Sigma), polyethylene glycol (PEG; Sigma), and N-acetylcysteine (Sigma) were diluted in PBS. PEG in catalase-PEG allows an increase in the stability and brain penetration of catalase.

Ibotenate activates *N*-methyl-D-aspartate (NMDA) and metabotropic glutamatergic receptors while S-bromowillardiine activates both $\alpha$-3-amino-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors [26,37,38]. N-acetylcysteine, catalase, and catalase-PEG were used as reference antioxidant agents with hydrogen peroxide scavenging properties [39,40].

### *Expression and purification of recombinant human PRDX5*

Human PRDX5 cDNA [30] was amplified by PCR using forward primer 5'-GCTGCAGGATCCGCCCCAATCAAGGTGGGAG-3' (*Bam*HI site underlined) and reverse primer 5'-GGCCCAAAGCTTCAGAGCTGTGAGATGATA-3' (*Hind*III site underlined). The PCR product was digested with *Bam*HI and *Hind*III and ligated into the pQE-30 expression vector (Qiagen, Valencia, CA, USA). The insert was sequenced, and the N-terminal fusion with the hexahistidine (6xHis) tag was confirmed. The resulting vector was used to transform *Escherichia coli* strain M15 (pRep4). *E. coli* was grown at 37°C in Luria Broth (LB) medium containing 1 mM isopropyl-$\beta$-D-thiogalactoside. Pelleted cells were sonicated and lysed in 10 mM imidazole, 50 mM phosphate, and 300 mM NaCl (pH 8) prior to centrifugation. The supernatant containing 6xHis-tagged PRDX5 was loaded onto a $Ni^{2+}$-NTA column (Qiagen). The column was washed and the protein was eluted with 50 mM phosphate, 300 mM NaCl, and 250 mM imidazole (pH 8). Eluted protein was then dialysed against PBS (pH 7.2). The mutated recombinant PRDX5, in which N-terminal catalytic $Cys^{47}$ [41] was replaced by a serine, was generated by standard PCR-mediated site-directed mutagenesis. Human PRDX5 cDNA cloned into pCR2.1 [30] was served as the template, and complementary primers containing a single-base mismatch that converted the codon Cys47 to a codon for Ser were used. The PCR product was ligated into the pQE-30 vector for transformation and expression as previously described.

The recombinant human PRDX5 used in this study corresponds to the 17 kDa PRDX5 identified in mammalian tissues [31] and human bronchoalveolar lavage fluid [35]. It contains the last 161 amino acids of the

C-terminal part of the protein as well as an hexahistidine tag at the N-terminus. It also contains the predicted peroxisomal addressing sequence and its catalytic site, but lacks the N-terminal mitochondrial addressing sequence [30]. The catalytic peroxidase activity of recombinant PRDX5 and the lack of activity of mutated PRDX5 were verified.

*Excitotoxic brain lesions*

We induced excitotoxic brain lesions by injecting ibotenate (10 μg) or S-bromowillardiine (15 μg) into developing mouse brains, as described previously [13,15,16, 26,42–47]. Briefly, anesthetized mouse pups were kept under a warming lamp to maintain body temperature. They were injected intracerebrally (into the neopallial parenchyma) with ibotenate or S-bromowillardiine on the fifth postnatal day (P5). Intracerebral injections were performed with a 25 gauge needle on a 50 μl Hamilton syringe mounted on a calibrated microdispenser. The needle was inserted 2 mm below the external surface of skin. The tip of the needle was placed in the frontoparietal area of the right hemisphere, 2 mm from the midline in the lateral-medial plane, and 3 mm from the bregma in the rostro-caudal plane. It was confirmed by histopathological observation that the tip of the needle always reached the periventricular white matter. Two 1 μl boluses of ibotenate or S-bromowillardiine were injected at 20 s intervals. The needle was left in place for an additional 20 s.

*Experimental groups*

Pups from at least two different litters were used in each experimental group, and data were obtained from two or more successive experiments.

In the first set of experiments designed to determine the neuroprotective action of PRDX5 against ibotenate-induced brain lesions (Figs. 1 and 2A), P5 pups received an intracerebral injection of ibotenate immediately followed by an intraperitoneal injection with recombinant PRDX5 (0.1, 1, 10, or 20 mg/kg) or mutated recombinant PRDX5 (the dose of 10 mg/kg was used as 10 mg/kg recombinant PRDX5 yielded maximum neuroprotection) diluted to a final volume of 5 μl.

In the second set of experiments designed to determine whether the neuroprotective effects of PRDX5 could be obtained following delayed systemic injection (Fig. 2B), P5 pups received an intracerebral injection of ibotenate followed by intraperitoneal administration of PBS alone, or 10 mg/kg PRDX5 at three different times: immediately (T0h), 4 (T4h), or 8 (T8h) h after ibotenate injection.

In the third series of experiments designed to compare the neuroprotective effects of reference antioxidant agents with PRDX5 (Figs. 2C–E), P5 pups received an intraparenchymal injection of ibotenate followed by intraperitoneal administration of one of the following drugs: catalase (6,000, 60,000, or 600,000 units/kg); catalase-PEG (6,000, 60,000, or 600,000 units/kg; 1 mg

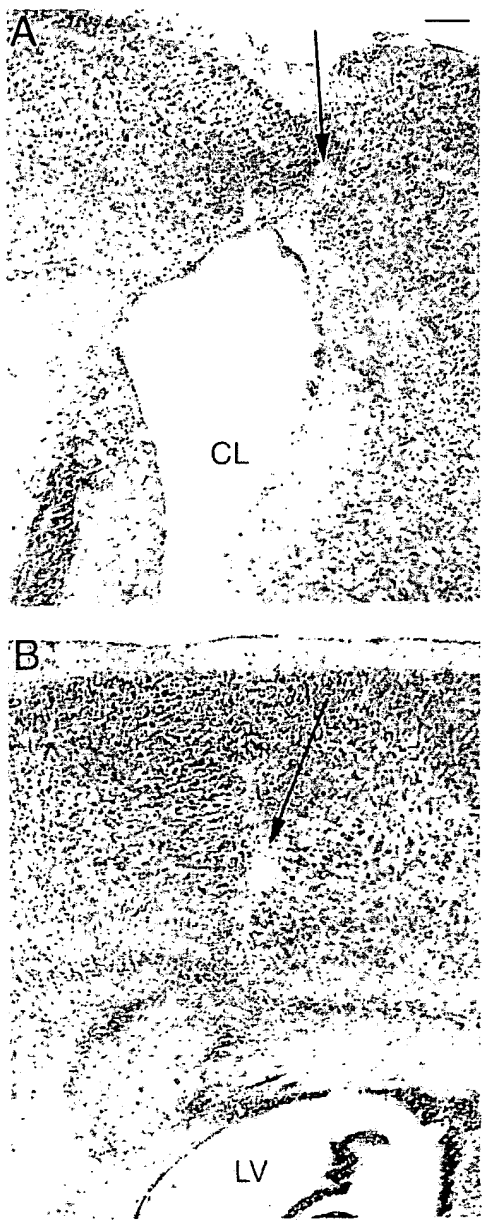


Fig. 1. Recombinant peroxiredoxin 5 (PRDX5) protects the newborn mouse brain against ibotenate-induced lesions. Cresyl violet-stained sections showing brain lesions induced by ibotenate injected at P5 and studied at the age of P10. (A) Brain from pup cotreated with intracerebral ibotenate and intraperitoneal phosphate buffer saline, showing the typical neuronal loss in layers II–VI (arrow) and the white matter cystic lesion (CL). (B) Brain from pup cotreated with intracerebral ibotenate and intraperitoneal 10 mg/kg PRDX5. LV-lateral ventricle. Bar: 40 μm.

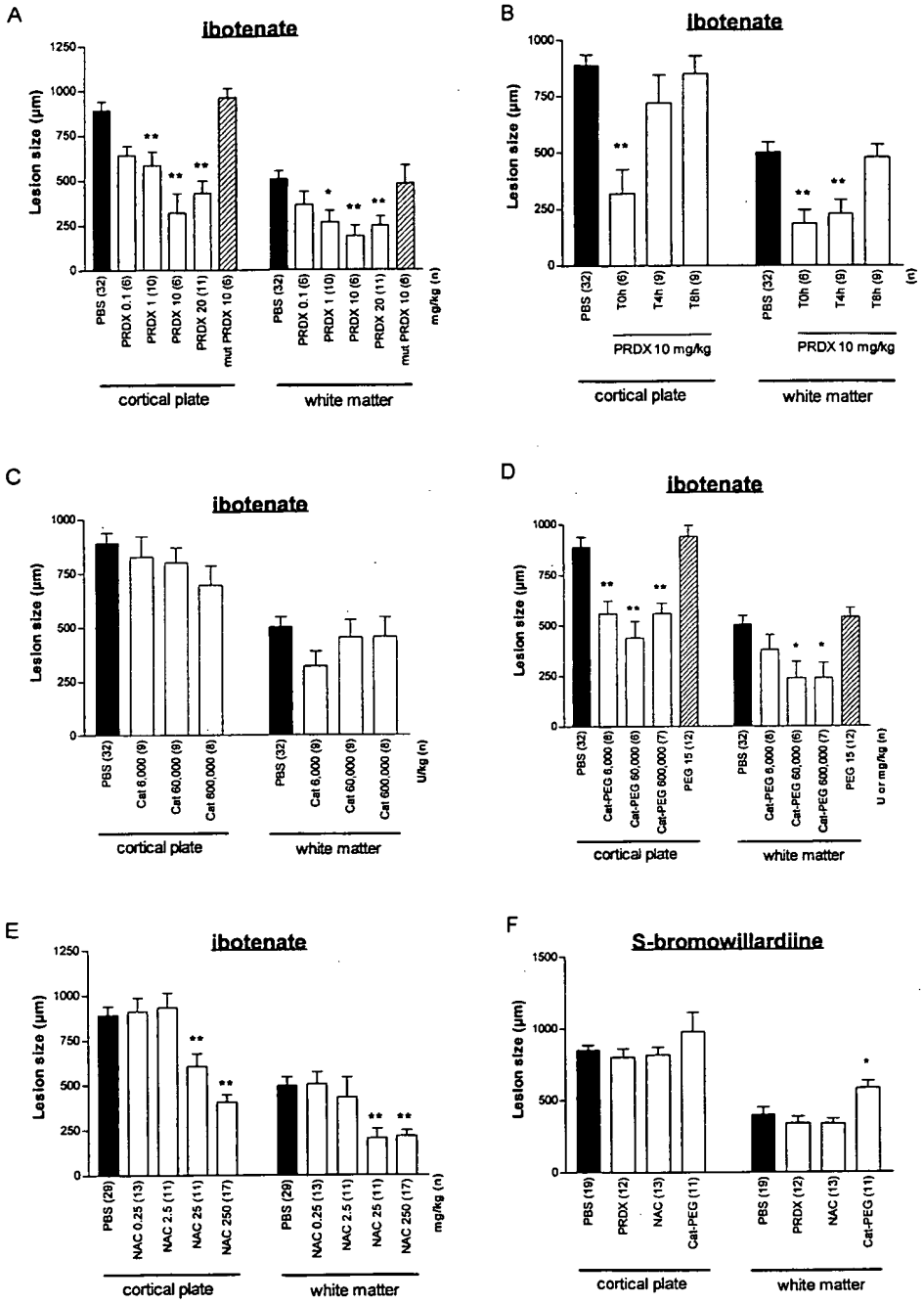


Fig. 2. Recombinant peroxiredoxin 5 (PRDX5) and reference antioxidant agents protect against ibotenate-induced brain lesions but not against S-bromowillardiine-induced lesions. (A) Effects of different doses of recombinant PRDX5 (PRDX) and recombinant mutated PRDX5 (mut PRDX) administered immediately after ibotenate on excitotoxic brain lesions. (B) Effects of delayed (time elapsed between ibotenate and PRDX5 injections is indicated on the *x* axis) administration of 10 mg/kg recombinant PRDX5 (PRDX) on ibotenate-induced lesions. (C) Effects of different doses of catalase (Cat) administered immediately after ibotenate on excitotoxic brain lesions. (D) Effects of different doses of catalase-polyethylene glycol (cat-PEG in U/kg) or of PEG (in mg/kg) administered immediately after ibotenate on excitotoxic brain lesions (E) Effects of different doses of N-acetylcysteine (NAC) administered immediately after ibotenate on excitotoxic brain lesions. (F) Effects of 10 mg/kg recombinant PRDX5 (PRDX), 250 mg/kg N-acetylcysteine (NAC) or 600,000 U/kg catalase-PEG (Cat-PEG) administered immediately after S-bromowillardiine on excitotoxic brain lesions. In all experiments, lesions were produced on P5 and analyzed on P10. Bars represent mean length of the lesions in the fronto-occipital axis ± SEM. Asterisks indicate difference from control (black bar); $*p < .05$, $**p < .01$, $***p < .001$ in analysis of variance with Dunnet's multiple comparison test (A, D–F) or in contrasts (B). Numbers in brackets are the numbers of animals used in each experimental group.

= 40,000 units); PEG (15 mg/kg; this dose of PEG is equal to the amount of PEG present in the highest dose catalase-PEG); N-acetylcysteine (0.25, 2.5, 25, or 250 mg/kg); or PBS alone.

In the fourth set of experiments designed to determine the neuroprotective action of PRDX5 against S-bromowillardiine-induced brain lesions (Fig. 2F), P5 pups received an intracerebral injection of S-bromowillardiine immediately followed by an intraperitoneal injection with one of the following drugs diluted to a final volume of 5 μl: recombinant PRDX5 (10 mg/kg); catalase-PEG (600,000 units/kg); N-acetylcysteine (250 mg/kg); or PBS alone.

*Determination of lesion size*

Mouse pups were sacrificed by decapitation 5 days after the excitotoxic challenge. Brains were fixed immediately in 4% formalin and remained in this solution for 5 days. Following paraffin embedding, we cut 16 μm thick coronal sections. Every third section was stained with cresyl-violet. The size of neocortical and white matter lesions can be defined by the length on three orthogonal axes: the lateral-medial axis (in a coronal plane), the radial axis (also in a coronal plane, from the pial surface to the lateral ventricle), and the fronto-occipital axis (in a sagittal plane). In previous studies [13,15,47], we found an excellent correlation among the measurements from the three axes of the excitotoxic lesions. Based on these findings, we cut serial sections of the entire brain in the coronal plane for this study. This permitted an accurate and reproducible determination of the sagittal fronto-occipital diameter (which is equal to the number of sections where the lesion was present multiplied by 16 μm). We used this measure as an index of the volume of the lesion. Numbers of brains analyzed in each experimental group are given in Fig. 2.

*Determination of in vivo cell death*

Pups were sacrificed at either 8 and 48 h after receiving intracerebral injections of ibotenate plus intraperitoneal injection of 10 mg/kg recombinant PRDX5 or ibotenate plus PBS. The brains were fixed in formalin and embedded in paraffin. Every third section was stained with cresyl violet. Sections adjacent to the most affected areas were selected for terminal transferase-mediated dUTP nick end labeling (Tunel), using an in situ cell death detection kit (Roche, Meylan, France) (Figs. 3A–B). Deparaffinized sections were treated with 20 mg/ml proteinase K for 20 min at 37°C and incubated for 2 min on ice with 0.1% Triton X-100. DNA strand breaks were identified by labeling of free 3'-OH termini with terminal deoxynucleotidyl transferase and fluorescein-labeled nucleotides. This procedure was run at 37°C for 60 min.

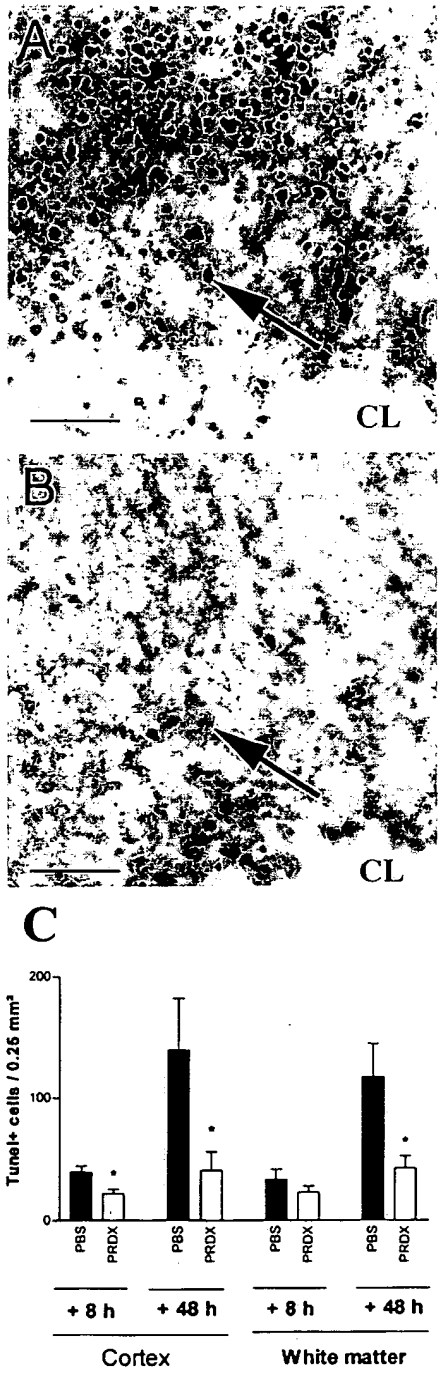


Fig. 3. Recombinant peroxiredoxin 5 (PRDX5) prevents ibotenate-induced neural cell death. (A,B) Terminal transferase-mediated dUTP end labeling (Tunel) stained sections showing cell death induced by ibotenate phosphate buffer saline (PBS). (A) or ibotenate 10 mg/kg recombinant PRDX5 (B) injected on P5 and studied on P6. PRDX-recombinant PRDX5; CL-cystic lesion in the white matter. Bar: 50 μm. (C) Quantitative analysis of Tunel-stained nuclei in the cortical plate and white matter lesions produced by ibotenate injection on P5 and studied 8 or 48 h later. Bars represent means ± SEM. Asterisks indicate difference from PBS group (*$p < .05$ in Student's $t$-test).

Incorporated nucleotides were detected using an anti-fluorescein antibody conjugated with alkaline phosphatase, and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate toluidonium salt as the substrates. Tunel-positive cells were counted in a 0.25 $mm^2$ area in the neocortical layers and underlying white matter at the level of ibotenate-induced lesions (Fig. 3C). Ten nonadjacent fields (separated by at least 50 $\mu$m to avoid counting the same nucleus twice) from five brains were studied in each group.

*Primary cultures of neurons*

Pregnant E14.5 mice were sacrificed using general anesthesia. Primary cultures of embryonic cortical neurons were prepared as described previously [48]. Briefly, the embryos were harvested, the brains removed, and the cortex dissociated both mechanically and chemically. Neurons were plated in 35 mm culture dishes previously coated with poly-DL-ornithine (30 $\mu$g/ml, Sigma) in Neurobasal supplemented with 2 mM glutamine and B27 (Life Technologies, Cergy Pontoise, France). They were plated at a density of $8.10^5$ cells/dish. Under these conditions, the neurons can survive and differentiate in the absence of an astroglial feeding layer. The cultures were maintained at 37°C in a humidified 95% air-5% $CO_2$ atmosphere. To inhibit proliferation of non-neuronal cells, 5 $\mu$M cytosine arabinoside (Sigma) was added after 3–6 days incubation. The mean astroglial cell density in mature cultures was very low (<5%). Only mature cultures (10–12 days incubation) were used for in vitro excitotoxic challenge.

*In vitro excitotoxic challenge and determination of neuronal cell death*

Neurons were incubated with 300 $\mu$M NMDA for 60 min in the presence of one of the following drugs or combination of drugs: 0.1, 1.0, 10, or 100 $\mu$M recombinant PRDX5; 10 $\mu$M dithiothreitol (DTT; Sigma); 10 $\mu$M recombinant PRDX5 + 10 $\mu$M DTT; or medium alone (control). DTT is not only a reductant, but it is also a nonphysiological electron donor for PRDX5 [30]. After replacing the medium, the neurons were incubated for an additional 8 h in the presence of drugs. Cells were then fixed in 4% paraformaldehyde and stained with bis-benzimide (Hoechst 33452, 10 $\mu$g/ml; Sigma), which labels nuclear chromatin. An observer who was blinded to treatment condition counted the nuclei featuring delayed cell death (e.g., pycnosis or chromatin condensation or fragmentation) (Fig. 4). Cell counting was done with a fluorescent microscope (UV-2A filter, excitation 370 nm, emission >400 nm; Zeiss, Oberkochen, Germany). Six to nine plates were used for each experimental group. The observer examined four to eight fields per

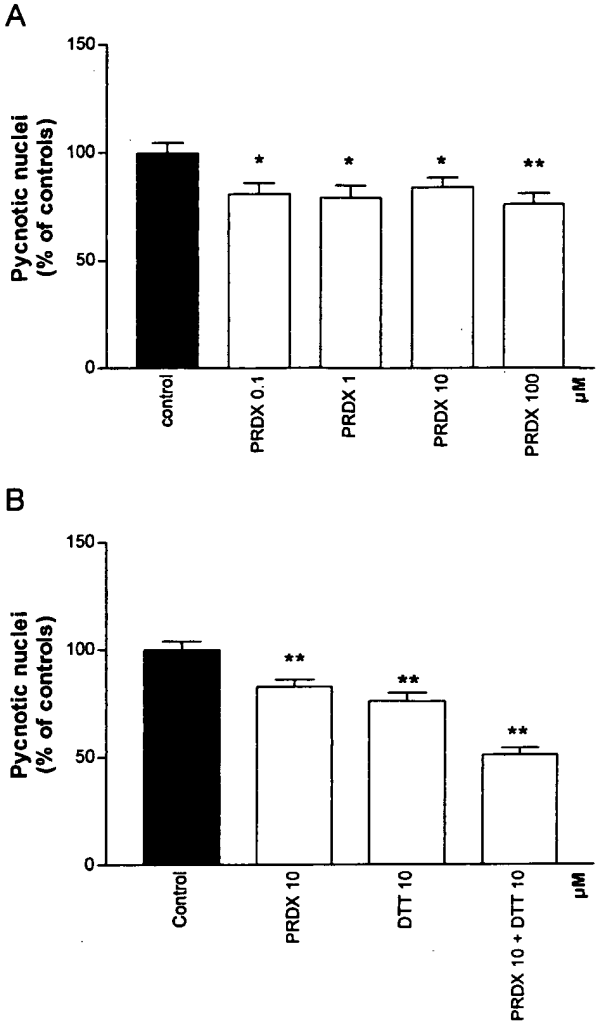


Fig. 4. Recombinant peroxiredoxin 5 (PRDX5) reduces in vitro excitotoxic neuronal death and this effect is potentiated by dithiothreitol. (A) Effect of different concentrations of recombinant PRDX5 (PRDX) on *N*-methyl-D-aspartate (NMDA)-induced (300 $\mu$M) in vitro neuronal cell death. (B) Effect of 10 $\mu$M recombinant PRDX5 (PRDX) alone or in conjunction with dithiothreitol (DTT) on NMDA-induced (300 $\mu$M) in vitro neuronal cell death. Quantitative analysis of cells showing apoptotic features (pycnotic nucleus) after staining with Bis-benzimide (Hoechst 33258). Number of pycnotic nuclei was divided by the total cell number and, for each experimental group, this ratio was normalized to ratio obtained in controls. Results are shown as means ± SEM. Asterisks indicate statistically significant differences between controls (phosphate buffer saline group) and experimental groups (*$p < 0.05$, **$p < 0.01$, ***$p < .001$ in analysis of variance with Dunnett's multiple comparison test).

plate, which contained 40–70 neurons. For each field, the ratio between the number of pycnotic nuclei and the total number of nuclei was calculated and used as an index of neuronal death. To take into account variation in cell viability across cultures, this ratio was normalized to the ratio obtained in control cultures exposed to NMDA alone (i.e., without recombinant PRDX5 or DTT), taken

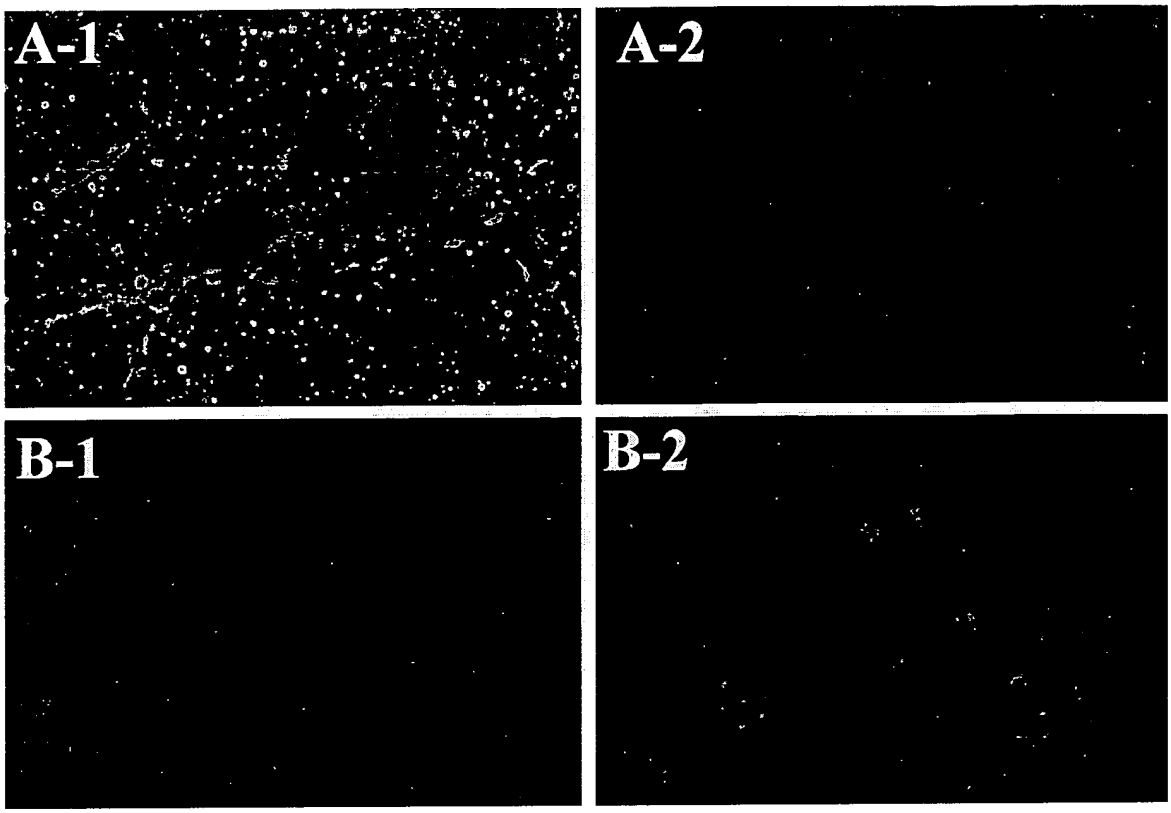


Fig. 5. Exogenous recombinant peroxiredoxin 5 (PRDX5) is detected in cultured neurons. Immunodetection of PRDX5 in neuronal cultures. Immunoreactivity for PRDX5 in neuronal cell cultures incubated during 30 min with 100 $\mu$M recombinant PRDX5 (A-1) or phosphate buffer saline (B-1). In (A-1); a cell showing a typical neuronal phenotype and incubated with recombinant PRDX5 is intensely labeled (arrow). (A-2) and (B-2) 4′,6-diamidino-2′phenylindole (DAPI) staining for nuclear visualization of cells immunostained in (A-1) and (B-1); respectively. Bar: 20 $\mu$m.

as 100% excitotoxicity. Two to three plates were used for each experimental condition. Each experiment was performed at least twice.

*Immunostaining for PRDX5 on cultured neurons*

For immunostaining experiments, embryonic cortical neurons were cultured on coverslips. The cells were incubated with 100 $\mu$M recombinant PRDX5 or PBS for 0.5, 1, 2, 3, 6, or 8 h, rinsed with PBS, and fixed with formalin-PBS (30 min). For immunostaining (Fig. 5), treated cells were rinsed three times with Tris-buffered solution (0.05 M, pH 7.6) containing 0.9% NaCl and 0.1% Triton X-100 (TBS-T), and immersed for 30 min in TBS-T containing 10% nonfat milk. Coverslips were then incubated overnight with rabbit antihuman PRDX5 diluted 1:200 in TBS-T containing 1% nonfat milk [36], followed by an incubation with fluorescein isothiocyanate conjugated donkey antirabbit IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) diluted 1/20 in TBS-T for 1 h. After each incubation, cells were washed twice for 10 min with TBST-T. The coverslips were mounted in Mowiol containing antifading reagent [1,4-diazabicyclo(2.2.2)octane, 25 mg/ml; Sigma] and 4′,6-diamidino-2′phenylindole (DAPI) (50 $\mu$g/ml; Roche, Basel, Switzerland) for nuclear staining. The coverslips were examined for fluorescein and DAPI by fluorescence microscopy with standard filters.

*Statistical analysis*

All variables were found normally distributed based on Skewness and Kurtosis analyses [49]. Most data were analyzed with a Student's *t*-test or a univariate analysis of variance (ANOVA) (GraphPad Prism version 3.03 for Windows, GraphPad Software). When significant difference was found among multiple experimental groups using ANOVA, multiple comparisons of treated groups against control (PBS) group were performed using Dunnett's post-hoc test [50]. In the subset of experiments where lesion size was evaluated at different time points after ibotenate injection, we used ANOVA with Treatment and Postinjection delay (Postinjection delay is defined as the time elapsed between injection and death) as

between-subject factors. When Postinjection delay by Treatment interaction was significant, we conducted comparisons between treated groups and control (PBS) group using Dunnett's post-hoc test.

## RESULTS

### *Clinical manifestations and mortality*

Overall, the mortality rate was low (<3%) in pups receiving intracerebral ibotenate or S-bromowillardiine injections. All death occurred within the first 48 h following the excitotoxic challenge, not permitting to include these brains in the histological analysis. All pups injected with ibotenate or S-bromowillardiine displayed tonic-clonic convulsions within the first 24 h following the excitotoxic challenge. Intraperitoneal administration of PRDX5, catalase, catalase-PEG, or N-acetylcysteine did not modify the mortality rate significantly (<3% in all experimental groups) nor did it alter the incidence, severity, or phenotype of convulsions.

### *Excitotoxic lesions and neuroprotection of recombinant PRDX5*

Pups injected intracerebrally with ibotenate and intraperitoneally with PBS on P5 developed cortical lesions and periventricular white matter cysts (Figs. 1A and 2A). The cortical lesion was typical, with severe neuronal loss in all neocortical layers and almost complete disappearance of neuronal cell bodies along the axis of ibotenate injection.

Intraperitoneal administration of recombinant PRDX5 immediately after ibotenate produced a dose-dependent U-shaped protective effect against the ibotenate-induced cortical plate and white matter lesions (Figs. 1B and 2A). In contrast, mutated recombinant PRDX5 had no detectable protective effect against ibotenate-induced lesions (Fig. 2A). When intraperitoneal injection of recombinant PRDX5 followed the excitotoxic challenge, the neuroprotective effect was also a function of time. Protection of the cortical plate was observed in groups receiving recombinant PRDX5 immediately after ibotenate while white matter protection was observed in groups receiving recombinant PRDX5 within the first 4 hours after ibotenate administration (Fig. 2B).

Administration of intracerebral ibotenate and intraperitoneal catalase-PEG or N-acetylcysteine mimicked the neuroprotective effects of recombinant PRDX5 against excitotoxic brain lesions (Figs. 2C–E). However, administration of catalase provided no detectable protection.

As noted previously [16,26], intracerebral injection of S-bromowillardiine also produced cortical plate lesions and periventricular white matter cysts (Fig. 2F). Intraperitoneal administration of recombinant PRDX5, catalase-PEG, or N-acetylcysteine had no detectable protective effect on S-bromowillardiine-induced brain lesions (Fig. 2F).

### *Effects of recombinant PRDX5 on excitotoxic neuronal cell death*

Tunel staining performed at 8 and 48 h after ibotenate injection showed that recombinant PRDX5 caused a significant reduction in cortical plate lesions (at both studied time points) and white matter cell death (at 48 h postibotenate) (Fig. 3).

### *Effects of recombinant PRDX5 on in vitro neuronal cell death*

When compared to cultures treated with PBS (controls), exposure to recombinant PRDX5 resulted in a moderate but significant reduction in NMDA-induced neuronal cell death (Fig. 4A). Interestingly, the addition of equimolar recombinant PRDX5 and DTT (an electron donor) led to a larger reduction in NMDA-induced neuronal cell death compared to the addition of PRDX5 or DTT alone (Fig. 4B).

### *Localization of administered recombinant PRDX5 in neuronal cultures*

A rabbit polyclonal antibody directed to PRDX5 was used to localize recombinant PRDX5 in neuronal cultures. Immunoreactivity was intense in cells incubated with recombinant PRDX5 for 30 min to 8 h (Fig. 5A-1), and weak in cells treated with PBS only (Fig. 5B-1). Immunoreactivity in PBS-treated cells reflected endogenous PRDX5. In cultures treated with PRDX5, cells with neuronal phenotype showed the most intense labeling. However, it should be noted that not all cells were immunoreactive (Fig. 5A).

## DISCUSSION

The present study provides experimental evidence that recombinant PRDX5 prevents excitotoxic lesions in the developing brain. In this mouse model, PRDX5 had a dose-dependent effect on the protection of the developing cortical plate and white matter against ibotenate but not against S-bromowillardiine. Similar neuroprotective effects were provided by catalase-PEG and N-acetylcysteine, two classical antioxidants.

Although ibotenate and S-bromowillardiine injections in newborn mice produce similar neuropathological patterns of brain lesions, the cellular and molecular mechanisms involved are quite distinct. As previously mentioned, ibotenate is a NMDA and metabotropic receptor agonist, while S-bromowillardiine activates AMPA-kai-

nate receptors [26,37,38]. Moreover, ibotenate injection leads to an early white matter microglial activation accompanied by an astrocytic cell death [16] while AMPA receptor activation induces an early white matter oligodendrocyte cell death [20]. Proinflammatory cytokines, such as interleukin-1-$\beta$, greatly exacerbate ibotenate-induced brain lesions, but have very limited effects on S-bromowillardiine-induced brain lesions [44] (Gressens P, unpublished results). Several neuroprotective drugs, including melatonin and brain-derived neurotrophic factor, have been shown to have very different protective effects against ibotenate and S-bromowillardiine insults [47].

The lack of neuroprotective effects from PRDX5, catalase-PEG, and acetylcysteine against S-bromowillardiine-induced brain lesions found in the present study does not necessarily mean that free radicals are not produced in excess following AMPA or AMPA-kainate receptor overactivation. We have demonstrated in previous experiments using this murine model that 8-alkylamino-1,4-benzoxazine antioxidant provides neuroprotection against S-bromowillardiine-induced lesions [26]. Furthermore, cells of the oligodendrocyte lineage are exquisitely sensitive to oxidative stress [51–53]. Putting it together, these data from the newborn mouse brain model suggest that hydrogen peroxide could be a major reactive species produced after NMDA receptor activation while other ROS may be more predominant after AMPA-kainate receptor activation. Further studies, perhaps incorporating dialysis [54,55], would be useful to confirm this hypothesis.

In primary cultures of cortical neurons, PRDX5 required the presence of DTT to exert a neuroprotective effect. DTT is not only a reductant, but also a nonphysiological electron donor for PRDX5. This finding from the present study supports previous in vitro data [30]. In contrast, in vivo experiments, PRDX5 was neuroprotective even in the absence of exogenous administration of DTT suggesting that either in situ neurons or neighboring cells, like astrocytes, are able to provide physiological electron donors for PRDX5 such as thioredoxin.

PRDX5 was initially isolated from broncho-alveolar extracellular fluid from patients with lung inflammation [35]. This finding suggests that PRDX5 might exert some of its antioxidant properties in the extracellular milieu. On the other hand, PRDX5 has peroxisomal and mitochondrial sorting signals. Previous in vitro studies have shown colocalization of PRDX5 with peroxisomal and mitochondrial proteins [30], suggesting a potential intracellular antioxidant role of PRDX5 in these organelles. In the present study, exogenously administered recombinant PRDX5 may have been able to quench extracellular free radicals at the site of ibotenate-induced brain lesions. The fact that we could detect exogenous PRDX5 in cultured neurons suggests that, at least in cell culture, extracellular PRDX5 is able to penetrate into some neurons. Further studies using confocal microscopy to determine the in vitro and in vivo subcellular localization of exogenous PRDX5 will be necessary to further elucidate this issue. It would also be interesting to produce and examine the full recombinant molecule, including the mitochondrial sorting signal, since mitochondria have been shown to be a major intracellular source of ROS, especially in pathological conditions [56–58].

Preterm neonates have a high susceptibility to oxidative stress for two reasons. (i) They are exposed to several pro-oxidant conditions, including brain reperfusion following hypoxic-ischemic insult, as well as high concentrations of inhaled oxygen and exogenous iron [59]. (ii) They have low levels of endogenous antioxidant molecules [60,61]. Several studies have found low levels of glutathione peroxidase, superoxide dismutase, vitamin E, and ceruloplasmin in neonatal plasma [62,63]. Furthermore, human preterm infants have a deficiency in cord blood superoxide dismutase and gluathione peroxidase [64]. To our knowledge, endogenous levels of peroxiredoxins, which could represent an additional line of intrinsic defence against oxidative stress, have not been evaluated in human newborns.

The present study demonstrates that recombinant PRDX5 is protective against excitotoxic murine brain lesions that are similar to damage seen in human cerebral palsy. PRDX5 may also be protective in other neurodegenerative conditions associated with oxidative stress, especially if related to NMDA receptor-mediated excitotoxicity.

*Acknowledgements* — We are grateful to Philippe Evrard for his support, and Leslie Scwhendimann and Bénédicte Vucic for their excellent technical assistance. We also thank Min-Xia Wang for reading the manuscript and his very helpful suggestions. This work was supported by the INSERM, the Académie Nationale de Médecine (France), the Fondation Grace de Monaco, the Association APRESAN, the Société Française de Médecine Périnatale, the Fonds National de la Recherche Scientifique (Belgique) and the Communauté Française de Belgique-Actions de Recherche Concertées.

## REFERENCES

[1] Hagberg, B.; Hagberg, G.; Olow, I.; van Wendt, L. The changing panorama of cerebral palsy in Sweden. VII. Prevalence and origin in the birth year period 1987–90. *Acta Paediatr.* **85**:954–960; 1996.

[2] Kuban, K. C.; Leviton, A. Cerebral palsy. *N. Engl. J. Med.* **330**:188–195; 1994.

[3] Inder, T. E.; Huppi, P. S.; Warfield, S.; Kikinis, R.; Zientara, G. P.; Barnes, P. D.; Jolesz, F.; Volpe, J. J. Periventricular white matter injury in the premature infant is followed by reduced cerebral cortical gray matter volume at term. *Ann. Neurol.* **46**: 755–760; 1999.

[4] Peterson, B. S.; Vohr, B.; Staib, L. H.; Cannistraci, C. J.; Dolberg, A.; Schneider, K. C.; Katz, K. H.; Westerveld, M.; Sparrow, S.; Anderson, A. W.; Duncan, C. C.; Makuch, R. W.; Gore, J. C.;

Ment, L. R. Regional brain volume abnormalities and long-term cognitive outcome in preterm infants. *JAMA* **284**:1939–1947; 2000.

[5] Marin-Padilla, M. Developmental neuropathology and impact of perinatal brain damage. III: gray matter lesions of the neocortex. *J. Neuropathol. Exp. Neurol.* **58**:407–429; 1999.

[6] Volpe, J. *Neurology of the newborn*, 4th ed. Philadelphia: W.B. Saunders; 2001.

[7] Nelson, K. B.; Ellenberg, J. H. Antecedents of cerebral palsy. Multivariate analysis of risk. *N. Engl. J. Med.* **315**:81–86; 1986.

[8] Dammann, O.; Leviton, A. Role of the fetus in perinatal infection and neonatal brain damage. *Curr. Opin. Pediatr.* **12**:99–104; 2000.

[9] Johnston, M. V. Excitotoxicity in neonatal hypoxia. *Ment. Retard. Dev. Disabil. Res. Rev.* **7**:229–234; 2001.

[10] Johnston, M. V.; Trescher, W. H.; Ishida, A.; Nakajima, W. Neurobiology of hypoxic-ischemic injury in the developing brain. *Pediatr. Res.* **49**:735–741; 2001.

[11] Inder, T.; Mocatta, T.; Darlow, B.; Spencer, C.; Volpe, J. J.; Winterbourn, C. Elevated free radical products in the cerebrospinal fluid of VLBW infants with cerebral white matter injury. *Pediatr. Res.* **52**:213–218; 2002.

[12] Inder, T.; Mocatta, T.; Darlow, B.; Spencer, C.; Senthilmohan, R.; Winterbourn, C. C.; Volpe, J. J. Markers of oxidative injury in the cerebrospinal fluid of a premature infant with meningitis and periventricular leukomalacia. *J. Pediatr.* **140**:617–621; 2002.

[13] Marret, S.; Mukendi, R.; Gadisseux, J. F.; Gressens, P.; Evrard, P. Effect of ibotenate on brain development: an excitotoxic mouse model of microgyria and posthypoxic-like lesions. *J. Neuropathol. Exp. Neurol.* **54**:358–370; 1995.

[14] Redecker, C.; Hagemann, G.; Witte, O. W.; Marret, S.; Evrard, P.; Gressens, P. Long-term evolution of excitotoxic cortical dysgenesis induced in the developing rat brain. *Brain Res. Dev. Brain Res.* **109**:109–113; 1998.

[15] Gressens, P.; Marret, S.; Hill, J. M.; Brenneman, D. E.; Gozes, I.; Fridkin, M.; Evrard, P. Vasoactive intestinal peptide prevents excitotoxic cell death in the murine developing brain. *J. Clin. Invest.* **100**:390–397; 1997.

[16] Tahraoui, S. L.; Marret, S.; Bodenant, C.; Leroux, P.; Dommergues, M. A.; Evrard, P.; Gressens, P. Central role of microglia in neonatal excitotoxic lesions of the murine periventricular white matter. *Brain Pathol.* **11**:56–71; 2001.

[17] McDonald, J. W.; Johnston, M. V. Neuroprotective synergism of 2-amino-3-phosphonoproprionate (D,L-AP3) and MK-801 against ibotenate induced brain injury. *Neurosci. Lett.* **145**:213–216; 1992.

[18] Silverstein, F. S.; Chen, R.; Johnston, M. V. The glutamate analogue quisqualic acid is neurotoxic in striatum and hippocampus of immature rat brain. *Neurosci. Lett.* **71**:13–18; 1986.

[19] Acarin, L.; Gonzalez, B.; Castellano, B.; Castro, A. J. Microglial response to N-methyl-D-aspartate-mediated excitotoxicity in the immature rat brain. *J. Comp. Neurol.* **367**:361–374; 1996.

[20] Follett, P. L.; Rosenberg, P. A.; Volpe, J. J.; Jensen, F. E. NBQX attenuates excitotoxic injury in developing white matter. *J. Neurosci.* **20**:9235–9241; 2000.

[21] Dugan, L. L.; Choi, D. W. Excitotoxicity, free radicals, and cell membrane changes. *Ann. Neurol.* **35**(Suppl):17–21; 1994.

[22] Volpe, J. J. Neurobiology of periventricular leukomalacia in the premature infant. *Pediatr. Res.* **50**:553–562; 2001.

[23] Martin, L. J.; Brambrink, A. M.; Price, A. C.; Kaiser, A.; Agnew, D. M.; Ichord, R. N.; Traystman, R. J. Neuronal death in newborn striatum after hypoxia-ischemia is necrosis and evolves with oxidative stress. *Neurobiol. Dis.* **7**:169–191; 2000.

[24] Tsuji, M.; Higuchi, Y.; Shiraishi, K.; Kume, T.; Akaike, A.; Hattori, H. Protective effect of aminoguanidine on hypoxic-ischemic brain damage and temporal profile of brain nitric oxide in neonatal rat. *Pediatr. Res.* **47**:79–83; 2000.

[25] Tan, S.; Zhou, F.; Nielsen, V. G.; Wang, Z.; Gladson, C. L.; Parks, D. A. Increased injury following intermittent fetal hypoxia-reoxygenation is associated with increased free radical production in fetal rabbit brain. *J. Neuropathol. Exp. Neurol.* **58**:972–981; 1999.

[26] Largeron, M.; Mesples, B.; Gressens, P.; Cecchelli, R.; Spedding, M.; Le Ridant, A.; Fleury, M. The neuroprotective activity of 8-alkylamino-1,4-benzoxazine antioxidants. *Eur. J. Pharmacol.* **424**:189–194; 2001.

[27] Marret, S.; Bonnier, C.; Raymackers, J. M.; Delpech, A.; Evrard, P.; Gressens, P. Glycine antagonist and NO synthase inhibitor protect the developing mouse brain against neonatal excitotoxic lesions. *Pediatr. Res.* **45**:337–342; 1999.

[28] Rhee, S. G.; Kang, S. W.; Chang, T. S.; Jeong, W.; Kim, K. Peroxiredoxin, a novel family of peroxidases. *IUBMB Life* **52**: 35–41; 2001.

[29] Hofmann, B.; Hecht, H. J.; Flohe, L. Peroxiredoxins. *Biol. Chem.* **383**:347–364; 2002.

[30] Knoops, B.; Clippe, A.; Bogard, C.; Arsalane, K.; Wattiez, R.; Hermans, C.; Duconseille, E.; Falmagne, P.; Bernard, A. Cloning and characterization of AOEB166, a novel mammalian antioxidant enzyme of the peroxiredoxin family. *J. Biol. Chem.* **274**: 30451–30458; 1999.

[31] Seo, M. S.; Kang, S. W.; Kim, K.; Baines, I. C.; Lee, T. H.; Rhee, S. G. Identification of a new type of mammalian peroxiredoxin that forms an intramolecular disulfide as a reaction intermediate. *J. Biol. Chem.* **275**:20346–20354; 2000.

[32] Kropotov, A.; Sedova, V.; Ivanov, V.; Sazeeva, N.; Tomilin, A.; Krutilina, R.; Oei, S. L.; Griesenbeck, J.; Buchlow, G.; Tomilin, N. A novel human DNA-binding protein with sequence similarity to a subfamily of redox proteins which is able to repress RNA-polymerase-III-driven transcription of the Alu-family retroposons in vitro. *Eur. J. Biochem.* **260**:336–346; 1999.

[33] Yamashita, H.; Avraham, S.; Jiang, S.; London, R.; Van Veldhoven, P. P.; Subramani, S.; Rogers, R. A.; Avraham, H. Characterization of human and murine PMP20 peroxisomal proteins that exhibit antioxidant activity in vitro. *J. Biol. Chem.* **274**: 29897–29904; 1999.

[34] Declercq, J. P.; Evrard, C.; Clippe, A.; Stricht, D. V.; Bernard, A.; Knoops, B. Crystal structure of human peroxiredoxin 5, a novel type of mammalian peroxiredoxin at 1.5 A resolution. *J. Mol. Biol.* **311**:751–759; 2001.

[35] Wattiez, R.; Hermans, C.; Bernard, A.; Lesur, O.; Falmagne, P. Human bronchoalveolar lavage fluid: two-dimensional gel electrophoresis, amino acid microsequencing and identification of major proteins. *Electrophoresis* **20**:1634–1645; 1999.

[36] Wang, M. X.; Wei, A.; Yuan, J.; Clippe, A.; Bernard, A.; Knoops, B.; Murrell, G. A. Antioxidant enzyme peroxiredoxin 5 is up-regulated in degenerative human tendon. *Biochem. Biophys. Res. Commun.* **284**:667–673; 2001.

[37] Zorumski, C. F.; Thio, L. L.; Clifford, D. B. Effects of bromowillardiine and willardiine on non-N-methyl-D-aspartate receptors in postnatal rat hippocampal neurons. *Mol. Pharmacol.* **40**: 45–51; 1991.

[38] Patneau, D. K.; Mayer, M. L.; Jane, D. E.; Watkins, J. C. Activation and desensitization of AMPA/kainate receptors by novel derivatives of willardiine. *J. Neurosci.* **12**:595–606; 1992.

[39] Liu, T. H.; Beckman, J. S.; Freeman, B. A.; Hogan, E. L.; Hsu, C. Y. Polyethylene glycol-conjugated superoxide dismutase and catalase reduce ischemic brain injury. *Am. J. Physiol.* **256**:H589–593; 1989.

[40] He, Y. Y.; Hsu, C. Y.; Ezrin, A. M.; Miller, M. S. Polyethylene glycol-conjugated superoxide dismutase in focal cerebral ischemia-reperfusion. *Am. J. Physiol.* **265**:H252–256; 1993.

[41] Chae, H. Z.; Uhm, T. B.; Rhee, S. G. Dimerization of thiol-specific antioxidant and the essential role of cysteine 47. *Proc. Natl. Acad. Sci. USA* **91**:7022–7026; 1994.

[42] Gressens, P.; Marret, S.; Martin, J. L.; Laquerriere, A.; Lombet, A.; Evrard, P. Regulation of neuroprotective action of vasoactive intestinal peptide in the murine developing brain by protein kinase C and mitogen-activated protein kinase cascades: in vivo and in vitro studies. *J. Neurochem.* **70**:2574–2584; 1998.

[43] Gressens, P.; Besse, L.; Robberecht, P.; Gozes, I.; Fridkin, M.; Evrard, P. Neuroprotection of the developing brain by systemic

administration of vasoactive intestinal peptide derivatives. *J. Pharmacol. Exp. Ther.* **288**:1207–1213; 1999.
[44] Dommergues, M. A.; Patkai, J.; Renauld, J. C.; Evrard, P.; Gressens, P. Proinflammatory cytokines and interleukin-9 exacerbate excitotoxic lesions of the newborn murine neopallium. *Ann. Neurol.* **47**:54–63; 2000.
[45] Laudenbach, V.; Calo, G.; Guerrini, R.; Lamboley, G.; Benoist, J. F.; Evrard, P.; Gressens, P. Nociceptin/orphanin FQ exacerbates excitotoxic white-matter lesions in the murine neonatal brain. *J. Clin. Invest.* **107**:457–466; 2001.
[46] Patkai, J.; Mesples, B.; Dommergues, M. A.; Fromont, G.; Thornton, E. M.; Renauld, J. C.; Evrard, P.; Gressens, P. Deleterious effects of IL-9-activated mast cells and neuroprotection by antihistamine drugs in the developing mouse brain. *Pediatr. Res.* **50**:222–230; 2001.
[47] Husson, I.; Mesples, B.; Bac, P.; Vamecq, J.; Evrard, P.; Gressens, P. Melatoninergic neuroprotection of the murine periventricular white matter against neonatal excitotoxic challenge. *Ann. Neurol.* **51**:82–92; 2002.
[48] de Lima, A. D.; Merten, M. D.; Voigt, T. Neuritic differentiation and synaptogenesis in serum-free neuronal cultures of the rat cerebral cortex. *J. Comp. Neurol.* **382**:230–246; 1997.
[49] Tabachnick, B. G.; Fidell, L. S., eds. *Using multivariate statistics*, 3rd ed. New York: Harper Collins; 1996.
[50] Dunnett, C. W. New tables for mutiple comparaisons with a control. *Biometrics* 482–491; 1964.
[51] Mouzannar, R.; Miric, S. J.; Wiggins, R. C.; Konat, G. W. Hydrogen peroxide induces rapid digestion of oligodendrocyte chromatin into high molecular weight fragments. *Neurochem. Int.* **38**:9–15; 2001.
[52] Back, S. A.; Gan, X.; Li, Y.; Rosenberg, P. A.; Volpe, J. J. Maturation-dependent vulnerability of oligodendrocytes to oxidative stress-induced death caused by glutathione depletion. *J. Neurosci.* **18**:6241–6253; 1998.
[53] Oka, A.; Belliveau, M. J.; Rosenberg, P. A.; Volpe, J. J. Vulnerability of oligodendroglia to glutamate: pharmacology, mechanisms, and prevention. *J. Neurosci.* **13**:1441–1453; 1993.
[54] Cambonie, G.; Laplanche, L.; Kamenka, J. M.; Barbanel, G. N-methyl-D-aspartate but not glutamate induces the release of hydroxyl radicals in the neonatal rat: modulation by group I metabotropic glutamate receptors. *J. Neurosci. Res.* **62**:84–90; 2000.
[55] Cambonie, G.; Kamenka, J. M.; Barbanel, G. Glutamate inhibition of NMDA-induced hydroxyl radical release: an ontogenic study in rat. *Neuroreport* **12**:2035–2039; 2001.
[56] Sanchez-Alvarez, R.; Almeida, A.; Medina, J. M. Oxidative stress in preterm rat brain is due to mitochondrial dysfunction. *Pediatr. Res.* **51**:34–39; 2002.
[57] Halliwell, B. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. *Drugs Aging* **18**:685–716; 2001.
[58] Moussaoui, S.; Obinu, M. C.; Daniel, N.; Reibaud, M.; Blanchard, V.; Imperato, A. The antioxidant ebselen prevents neurotoxicity and clinical symptoms in a primate model of Parkinson's disease. *Exp. Neurol.* **166**:235–245; 2000.
[59] Buonocore, G.; Perrone, S.; Bracci, R. Free radicals and brain damage in the newborn. *Biol. Neonate* **79**:180–186; 2001.
[60] Baydas, G.; Karatas, F.; Gursu, M. F.; Bozkurt, H. A.; Ilhan, N.; Yasar, A.; Canatan, H. Antioxidant vitamin levels in term and preterm infants and their relation to maternal vitamin status. *Arch. Med. Res.* **33**:276–280; 2002.
[61] Thibeault, D. W. The precarious antioxidant defenses of the preterm infant. *Am. J. Perinatol.* **17**:167–181; 2000.
[62] Haga, P.; Lunde, G. Selenium and vitamin E in cord blood from preterm and full term infants. *Acta Paediatr. Scand.* **67**:735–739; 1978.
[63] Bracci, R.; Buonocore, G.; Talluri, B.; Berni, S. Neonatal hyperbilirubinemia. Evidence for a role of the erythrocyte enzyme activities involved in the detoxification of oxygen radicals. *Acta Paediatr. Scand.* **77**:349–356; 1988.
[64] Inder, T. E.; Graham, P.; Sanderson, K.; Taylor, B. J. Lipid peroxidation as a measure of oxygen free radical damage in the very low birthweight infant. *Arch. Dis. Child Fetal Neonatal Ed.* **70**:F107–F111; 1994.

## ABBREVIATIONS

AMPA—α-3-amino-hydroxy-5-methyl-4-isoxazole propionic acid
CNS—central nervous system
DTT—dithiothreitol
NMDA—*N*-methyl-D-aspartate
PBS—phosphate buffer saline
PCR—polymerase chain reaction
PEG—polyethylene glycol
PRDX5—peroxiredoxin 5
PVL—periventricular leukomalacia
RNS—reactive nitrogen species
ROS—reactive oxygen species
TUNEL—terminal transferase-mediated dUTP

ATTACHMENT B

UNITED STATES PATENT AND TRADEMARK OFFICE

COMMISSIONER FOR PATENTS
UNITED STATES PATENT AND TRADEMARK OFFICE
WASHINGTON, D.C. 20231
www.uspto.gov

Date: February 21, 2002

To: Patent Examining Corps
Technology Center Directors

From: Stephen G. Kunin
Deputy Commissioner for Patent Examination Policy

Subject: Procedures for Relying on Facts Which are Not of Record as Common Knowledge or for Taking Official Notice

This memorandum clarifies the circumstances in which it is appropriate to take official notice of facts not in the record or to rely on "common knowledge" in making a rejection.

Recent court decisions have affected the Office's practice of taking official notice of facts by relying on common knowledge in the art without a reference. Specifically, the Supreme Court recently changed the standard of review applied to decisions of the Board of Patent Appeals and Interferences and the Trademark Trial and Appeal Board on appeal to the U.S. Court of Appeals for the Federal Circuit. *Dickinson v. Zurko,* 527 U.S. 150, 50 USPQ2d 1930 (1999). As a result, the Federal Circuit now reviews findings of fact under the "substantial evidence" standard under the Administrative Procedure Act (APA), rather than the former "clearly erroneous" standard. *In re Gartside,* 203 F.3d 1305, 1315, 53 USPQ2d 1769, 1775 (Fed. Cir. 2000).[1] This change in the review standard has affected the Federal Circuit's view of when the court or the USPTO may take notice of facts without specific documentary evidence support.[2]

On remand from the Supreme Court, the Federal Circuit in *In re Zurko,* 258 F.3d 1379, 59 USPQ2d 1693 (Fed. Cir. 2001), reversed the Board's decision upholding a rejection under 35 U.S.C. 103 for lack of substantial evidence. Specifically, in *Zurko* and other recent decisions, the court criticized the USPTO's reliance on "basic knowledge" or "common sense" to support an obviousness rejection, where there was no evidentiary support in the record for such a finding.[3] In light of the recent Federal Circuit decisions and the substantial evidence standard of review now applied to USPTO Board decisions, the following guidance is provided in order to assist the examiners in determining when it is appropriate to take official notice of facts without

supporting documentary evidence or to rely on common knowledge in the art in making a rejection, and if such official notice is taken, what evidence is necessary to support the examiner's conclusion of common knowledge in the art.

(1) Determine when it is appropriate to take official notice without documentary evidence to support the examiner's conclusion.

Official notice without documentary evidence to support an examiner's conclusion is permissible only in some circumstances. While "official notice" may be relied on, as noted in MPEP § 2144.03, these circumstances should be rare when an application is under final rejection or action under 37 CFR 1.113. Official notice unsupported by documentary evidence should only be taken by the examiner where the facts asserted to be well-known, or to be common knowledge in the art are capable of instant and unquestionable demonstration as being well-known.[4] In appropriate circumstances, it might not be unreasonable to take official notice of the fact that it is desirable to make something faster, cheaper, better, or stronger without the specific support of documentary evidence. Furthermore, it might not be unreasonable for the examiner in a first Office action to take official notice of facts by asserting that certain limitations in a dependent claim are old and well known expedients in the art without the support of documentary evidence provided the facts so noticed are of notorious character and serve only to "fill in the gaps" which might exist in the evidentiary showing made by the examiner to support a particular ground of rejection.[5]

It would not be appropriate for the examiner to take official notice of facts without citing a prior art reference where the facts asserted to be well known are not capable of instant and unquestionable demonstration as being well-known. For example, assertions of technical facts in the areas of esoteric technology or specific knowledge of the prior art must always be supported by citation to some reference work recognized as standard in the pertinent art.[6]

It is never appropriate to rely solely on "common knowledge" in the art without evidentiary support in the record, as the principal evidence upon which a rejection was based.[7] As the court held in *Zurko,* an assessment of basic knowledge and common sense that is not based on any evidence in the record lacks substantial evidence support.[8]

(2) If official notice is taken of a fact, unsupported by documentary evidence, the technical line of reasoning underlying a decision to take such notice must be clear and unmistakable.

Ordinarily, there must be some form of evidence in the record to support an assertion of common knowledge.[9] In certain older cases, official notice has been taken of a fact that is asserted to be "common knowledge" without specific reliance on documentary evidence where the fact noticed was readily verifiable, such as when other references of record supported the noticed fact, or where there

was nothing of record to contradict it.[10] If such notice is taken, the basis for such reasoning must be set forth explicitly. The examiner must provide specific factual findings predicated on sound technical and scientific reasoning to support his or her conclusion of common knowledge.[11] The applicant should be presented with the explicit basis on which the examiner regards the matter as subject to official notice and be allowed to challenge the assertion in the next reply after the Office action in which the common knowledge statement was made.

(3) If applicant challenges a factual assertion as not properly officially noticed or not properly based upon common knowledge, the examiner must support the finding with adequate evidence.

To adequately traverse such a finding, an applicant must specifically point out the supposed errors in the examiner's action, which would include stating why the noticed fact is not considered to be common knowledge or well-known in the art.[12] A general allegation that the claims define a patentable invention without any reference to the examiner's assertion of official notice would be inadequate. If applicant adequately traverses the examiner's assertion of official notice, the examiner must provide documentary evidence in the next Office action if the rejection is to be maintained.[13] If the examiner is relying on personal knowledge to support the finding of what is known in the art, the examiner must provide an affidavit or declaration setting forth specific factual statements and explanation to support the finding. See 37 CFR 1. l04(d)(2).

If applicant does not traverse the examiner's assertion of official notice or applicant's traverse is not adequate, the examiner should clearly indicate in the next Office action that the common knowledge or well-known in the art statement is taken to be admitted prior art because applicant either failed to traverse the examiner's assertion of official notice or that the traverse was inadequate. If the traverse was inadequate, the examiner should include an explanation as to why it was inadequate.

(4) Determine whether the next Office action should be made final.

If the examiner adds a reference in the next Office action after applicant's rebuttal, and the newly cited reference is added only as directly corresponding evidence to support the prior common knowledge finding, and it does not result in a new issue or constitute a new ground of rejection, the Office action may be made final. If no amendments are made to the claims, the examiner must not rely on any other teachings in the reference if the rejection is made final. If the newly cited reference is added for reasons other than to support the prior common knowledge statement and a new ground of rejection is introduced by the examiner that is not necessitated by applicant's amendment of the claims, the rejection may not be made final. See MPEP § 706.07(a).

(5) Summary.

Any rejection based on assertions that a fact is well-known or is common knowledge in the art without documentary evidence to support the examiner's conclusion should be judiciously applied. Furthermore, as noted by the court in *Ahiert,* any facts so noticed should be of notorious character and serve only to "fill in the gaps" in an insubstantial manner which might exist in the evidentiary showing made by the examiner to support a particular ground for rejection. It is never appropriate to rely solely on common knowledge in the art without evidentiary support in the record as the principal evidence upon which a rejection was based.[14]

MPEP § 2144.03 will be revised accordingly in the upcoming revision to be consistent with this memo.

Cc: Nicholas Godici
Esther Kepplinger
Kay Kim
David Lacey

[1] The Supreme Court has described substantial evidence review in the following manner:

> Substantial evidence is more than a mere scintilla. It means such relevant evidence as a reasonable mind might accept as adequate to support a conclusion...Mere uncorroborated hearsay or rumor does not constitute substantial evidence.

*Consolidated Edison Co. v. NLRB,* 305 U.S. 197, 229-30 (1938)(quoted in *Gartside,* 203 F.3d at 1312, 53 USPQ2d at 1773). "'Substantial evidence' review involves examination of the record as a whole, taking into account evidence that both justifies and detracts from an agency's decision." *Gartside,* 203 F.3d at 1312, 53 USPQ2d at 1773 (citing *Universal Camera Corp. v. NLRB,* 340 U.S. 474, 487-88 (1951)). Furthermore, the Supreme Court has also recognized that "the possibility of drawing two inconsistent conclusions from the evidence does not prevent an administrative agency's finding from being supported by substantial evidence." *Consolo v. Federal Maritime Comm'n,* 383 U.S. 607, 620 (1966) (quoted in *Gartside,* 203 F.3d at 1312, 53 USPQ2d at 1773).

[2] See *Packard Press, Inc. v. Hewlett~Packard Co.,* 227 F.3d 1352, 1360, *56* USPQ2d 1351, 1356 (Fed. Cir. 2000) (questioning authority to take judicial notice for the first time on appeal in light of the APA standard of review established by *Dickinson v. Zurko,* 527 U.S. at 165, 50 USPQ2d at 1937). Although the substantial evidence standard is deferential to the agency's decision, it imposes certain evidentiary requirements that must be met by the agency in formulating a decision. The Federal Circuit explained that "[i]n appeals from the Board, we have before us a comprehensive record that contains the arguments and evidence presented by the parties, including all of the relevant information upon which the board relied in rendering its decision." *Gartside,* 203 F.3d at 1314, 53 USPQ2d at 1774. Furthennore, the record is "closed, in that the Board's decision must be justified within the four corners of that record." *Id* Thus, the record before the USPTO "dictates the parameters of review" available to the court. *Id.* Accordingly, "the Board's opinion must explicate its factual conclusions, enabling [the court} to verify readily whether those conclusions are indeed supported by 'substantial evidence' contained within the record." *Id* (citing *Gechter v. Davidson,* 116 F.3d 1454, 1460, 43 USPQ2d 1030, 1035 (Fed. Cir. 1997)).

[3] *Zurko,* 258 F.3d at 1385, 59 USPQ2d 1697 ("the Board cannot simply reach conclusion based on its own understanding or experience—or on its assessment of what would be basic knowledge or common sense. Rather, the Board must point to some concrete evidence in the record in support of these findings."). See also *In re Lee,* __F.3d__ , 61USPQ2d 1430, 1434 (Fed. Cir. 2002) (The Board determined that it was not necessary to present a source of a teaching, suggestion, or motivation to combine the references because the conclusion of obviousness may be made from common knowledge and common sense of a person of ordinary skill in the art. The court reversed the Board's decision in sustaining a rejection under 35 U.S.C. 103 and stated that "common knowledge and common sense' on which the Board relied in rejecting Lee's application are not the specialized knowledge and expertise contemplated by the Administrative Procedure Act. Conclusory statements such as

those here provided do not fulfill the agency's obligation.. .The board cannot rely on conclusory statements when dealing with particular combinations of prior art and specific claims, but must set forth the rationale on which it relies").

[4] As noted by the court in *In re Ahlert,* 424 F.2d 1088, 1091, *165* USPQ 418, 420 (CCPA 1970), the notice of facts beyond the record which may be taken by the examiner must be "capable of such instant and unquestionable demonstration as to defy dispute" (citing *In re Knapp Monarch Co.,* 296 F.2d 230, 132 USPQ 6 (CCPA 1961)). In *Ahlert,* the court held that the Board properly took judicial notice that "it is old to adjust intensity of a flame in accordance with the heat requirement" See also *In re Fox,* 471, F.2d 1405, 1407, 176 USPQ 340, 341 (CCPA 1973) (the court took "judicial notice of the fact that tape recorders commonly erase tape automatically when new 'audio information' is recorded on a tape which already has a recording on it").

[5] *Zurko,* 258 F.3d at 1385, 59 USPQ2d at 1697; *In re Ahlert,* 424 F.2d at 1092, 165 USPQ at 421.

[6] *In re Ahiert,* 424 F.2d at 1091, 165 USPQ at 420-21. See also *In re Grose, 592* F.2d 1161, 1167-68, 201 USPQ *57,* 63 (CCPA 1979) ("[w]hen the PTO seeks to rely upon a chemical theory, in establishing a prima facie case of obviousness, it must provide evidentiary support for the existence and meaning of that theory."); *In re Eynde,* 480 F.2d 1364, 1370, 178 USPQ 470, 474 (CCPA 1973) ("we reject the notion that judicial or administrative notice may be taken of the state of the art. The facts constituting the state of the art are normally subject to the possibility of rational disagreement among reasonable men and are not amenable to the taking of such notice.").

[7] *Zurko,* 258 F.3d at 1385, 59 USPQ2d at 1697. While the court explained that, "as an administrative tribunal the Board clearly has expertise in the subject matter over which it exercises jurisdication," it make clear that such "expertise may provide sufficient support for conclusions [only] as to peripheral issue." *Id* at 13 85-86, *59* USPQ2d at 1697.

[8] *Zurko,* 258 F.3d at 1385, 59 USPQ2d at 1697. See also *In re Lee,* _F.3d at _61 USPQ2d at 1435.

[9] See *In re Lee,* _F.3d _at_, 61 USPQ2d 1434-35; *In re Zurko,* 258 F.3d at 1386, 59 USPQ2d at 1697 (holding that general conclusions concerning what is "basic knowledge" or "common sense" to one of ordinary skill in the art without specific factual findings and some concrete evidence in the record to support these findings will not support an obviousness rejection).

[10] See *In re Soli,* 317 F.2d 941, 945-46, 137 USPQ 797, 800 (CCPA 1963) (the court accepted the examiner's assertion that the use of "a control is standard procedure throughout the entire field of bacteriology" because it was readily verifiable and disclosed in references of record not cited by the Office); *In re Chevenard,* 139 F.2d 711, 713, 60 USPQ 239, 241 (CCPA 1943) (accepting examiner's finding that a brief heating at a higher temperature was the equivalent of a longer heating at a lower temperature where there was nothing in the record to indicate the contrary and where the applicant never demanded that the examiner produce evidence to support his statement).

[11] See *Soli,* 317 F.2d at 946, 37 USPQ at 801; *Chevenard,* 139 F.2d at 713, 60 USPQ at 241.

[12] See 37 CFR 1.111(b). See also *Chevenard,* 139 F.2d at 713, 60 USPQ at 241 ("[I]n the absence of any demand by appellant for the examiner to produce authority for his statement, we will not consider this contention.").

[13] See 37 CFR 1.104(c)(2). See also *Zurko,* 258 F.3d at 1386, 59 USPQ2d at 1697 ("the Board [or examiner] must point to some concrete evidence in the record in support of these findings" to satisfy the substantial evidence test).

[14] See *Zurko,* 258 F.3d at 1386, *59* USPQ2d at 1697; *Ahlert,* 424 F.2d at 1092, 165 USPQ 421.

# ATTACHMENT C

ROYAUME DE BELGIQUE

MINISTERE DES AFFAIRES ECONOMIQUES

# BREVET D'INVENTION

NUMERO DE PUBLICATION : 1011331A6

NUMERO DE DEPOT : 09700692

Classif. Internat. : C07K

Date de délivrance le : 06 Juillet 1999

---

**Le Ministre des Affaires Economiques,**

Vu la loi du 28 Mars 1984 sur les brevets d'invention, notamment l'article 22;

Vu l' arrêté royal du 2 Décembre 1986 relatif à la demande, à la délivrance et au maintien en vigueur des brevets d'invention, notamment l'article 28;

Vu le procès verbal dressé le 20 Août 1997 à 15H20 à l 'Office de la Propriété Industrielle

# ARRETE:

ARTICLE 1.- Il est délivré à : UNIVERSITE CATHOLIQUE DE LOUVAIN Halles Universitaires; UNIVERSITE DE MONS-HAINAUT place de l'Université 1, B-1348 LOUVAIN-LA-NEUVE(BELGIQUE); place du Parc 20, B-7000 MONS (BELGIQUE)

représenté(e)(s) par : VAN MALDEREN Joëlle, OFFICE VAN MALDEREN, Place Reine Fabiola 6/1 - B 1083 BRUXELLES.

un brevet d' invention d' une durée de 6 ans, sous réserve du paiement des taxes annuelles, pour : POLYPEPTIDE ASSOCIE AU PEROXYSOME, SEQUENCE NUCLEOTIDIQUE ENCODANT LEDIT POLYPEPTIDE ET LEUR UTILISATION DANS LE DIAGNOSTIC ET/OU LE TRAITEMENT DE MALADIES OU DE LESIONS PULMONAIRES.

INVENTEUR(S) : Knoops Bernard, rue Chapelle Notre-Dame 3/1, B-1341 Ceroux-Mousty (BE); Hermans Cedric, avenue des Glycines 42, B-1030 Bruxelles (BE); Bernard Alfred, avenue de la Chapelle 6, B-1200 Bruxelles (BE); Wattiez Ruddy, chemin du Sauvelon 17, B,7022 Hion (BE); Falmagne Paul, rue Point du Jour 8, B-7022 Mefvin (BE)

ARTICLE 2.- Ce brevet est délivré sans examen préalable de la brevetabilité de l'invention, sans garantie du mérite de l'invention ou de l'exactitude de la description de celle-ci et aux risques et périls du(des) demandeurs(s).

Bruxelles, le 06 Juillet 1999
PAR DELEGATION SPECIALE :

L. [illegible]
CONSEILLER

# POLYPEPTIDE ASSOCIE AU PEROXYSOME, SEQUENCE NUCLEOTIDIQUE ENCODANT LEDIT POLYPEPTIDE ET LEUR UTILISATION DANS LE DIAGNOSTIC ET/OU LE TRAITEMENT DE MALADIES OU DE LESIONS PULMONAIRES

## Objet de l'invention

La présente invention est relative à un nouveau polypeptide associé au peroxysome, la séquence nucléotidique encodant ledit polypeptide et les fractions de celle-ci ainsi que leur utilisation pour le diagnostic et/ou le traitement de maladies ou de lésions pulmonaires.

## Arrière-plan technologique à la base de l'invention

Les peroxysomes, également dénommés "microbodies", sont des organites intracellulaires qui diffèrent des mitochondries et des chloroplastes présents dans les cellules eukaryotes. Ces organites ne comprennent ni génome, ni ribosomes, mais contiennent certaines enzymes essentielles à différents processus cataboliques et anaboliques. Certaines de ces enzymes sont exprimées de manière continuelle tandis que d'autres sont induites dans certaines conditions appropriées.

Les peroxysomes effectuent un certain nombre de réactions essentielles telles que l'oxydation et la

respiration peroxysomale, la ß-oxydation des acides gras, le métabolisme du cholestérol et du dolichol, la synthèse des éthers phospholipides, le métabolisme du glyoxylate et le métabolisme de l'acide pipécolique.

Le métabolisme de l'oxydation peroxysomale comprend la formation de peroxyde d'hydrogène par un certain nombre d'oxydases et sa décomposition par une catalase.

Ces réactions sont responsables de la consommation de 20% de l'oxygène dans le foie. Différentes oxydases ont été identifiées dans les peroxysomes. L'élimination d'éthanol via la catalase dans le peroxysome et les processus d'oxydation via une déshydrogénase semblent être également des processus biochimiques importants.

Le système de ß-oxydation peroxysomale catalyse la ß-oxydation des chaînes courtes d'un certain nombre de dérivés d'acides gras qui ne peuvent être traités par les mitochondries. Celle-ci inclut l'oxydation des très longues chaînes d'acides gras, des acides di- ou trihydroxycholestanoïques, de l'acide pristanique, des longues chaînes d'acide dicarboxylique, de certaines prostaglandines, de certaines leukotriènes, des acides 12- et 15-hydroxyeicosatétraéonique, ainsi que de certains acides mono- et polyinsaturés. Le dosage des trois premiers composants est corrélé au diagnostic direct de certains désordres peroxysomaux.

Le peroxysome joue également un rôle essentiel dans la synthèse du cholestérol et d'autres isoprénoïdes. Les fibroblastes de patients affectés par un désordre de la biogenèse du peroxysome montrent une capacité inadéquate à synthétiser le cholestérol.

En outre, certains facteurs prolifératifs de peroxysomes provoquent une augmentation de l'incidence des tumeurs du foie dans certaines espèces.

Différents mécanismes ont été proposés pour la formation des hépato-carcinomes par des composants responsables de la prolifération peroxysomale, combinée à l'induction d'un stress oxydatif.

Par conséquent, l'identification de nouvelles molécules associées aux peroxysomes est d'une grande importance pour développer des outils diagnostiques et éventuellement des applications thérapeutiques dans le traitement de différentes maladies associées à des éventuelles déficiences de ces molécules.

De même, il est particulièrement utile d'identifier d'autres molécules présentes au niveau de certains organes, en particulier le poumon, et d'étudier leur association avec certaines pathologies, en particulier des maladies ou des lésions pulmonaires.

## Eléments caractéristiques de l'invention

Les Inventeurs ont identifié chez l'homme, dans un lavage alvéolaire des poumons, un nouveau polypeptide dont la séquence nucléotidique et en acides aminés a été caractérisée. Ce nouveau polypeptide ou protéine a été dénommé protéine B18.

Cette molécule présente certaines homologies avec des protéines peroxysomales de la levure et possède un tripeptide carboxyterminal SQL connu pour cibler et faciliter la translocation de certaines protéines au niveau du peroxysome.

L'objet de la présente invention est relatif à toute séquence d'acides nucléiques présentant plus de

70%, avantageusement plus de 85%, de préférence plus de 95% d'homologie avec la séquence SEQ ID NO 1 ou son brin complémentaire décrits ci-après.

La présente invention concerne également la séquence nucléotidique SEQ ID NO 1, son brin complémentaire ou des portions de ceux-ci (figure 5).

On entend par "portions de la séquence SEQ ID NO 1", toute séquence nucléotidique de plus de 15 paires de base susceptible d'identifier ou reconstituer (de préférence par amplification génétique) la séquence SEQ ID NO 1. De telles méthodes d'identification ou de reconstitution sont basées sur la technique d'hybridation, de préférence dans des conditions stringentes, par des sondes marquées (par un élément radioactif, par une enzyme, par un marqueur fluorescent, etc.) ou sur la technique de l'amplification génétique par l'emploi d'une ou plusieurs amorces spécifiques d'au moins 15 nucléotides, permettant d'identifier la séquence SEQ ID NO 1 et/ou de la reconstituer par des techniques d'amplification génétique bien connues de l'homme de l'art, en particulier les technologies PCR, LCR, CPR, etc.

Un autre aspect de la présente invention concerne la séquence d'acides aminés encodée par les séquences nucléotidiques telles que définies ci-dessus.

La présente invention concerne également une séquence d'acides aminés présentant plus de 70%, avantageusement plus de 85%, de préférence plus de 95%, d'homologie avec la séquence SEQ ID NO 1 (figure 5).

Un autre aspect de la présente invention est relatif à un polypeptide dont la séquence d'acides aminés est la séquence SEQ ID NO 1 ou une portion de celle-ci.

On entend par "portion de la séquence SEQ ID NO 1", un fragment de la séquence SEQ ID NO 1 ayant subi une ou plusieurs délétions tout en conservant plus de 85%, de préférence plus de 95%, de son activité biochimique.

De préférence, ladite séquence polypeptidique SEQ ID NO 1 possède un PI de 7.3 et un poids moléculaire de 17000 D, tels que définis après une électrophorèse bidimensionnelle.

L'invention concerne également les anticorps, y compris des fragments de ceux-ci tels que les extrémités hypervariables Fab, ..., dirigés contre la séquence nucléotidique et peptidique selon l'invention.

Un autre aspect de la présente invention concerne un dispositif de diagnostic tel qu'une trousse de diagnostic ou une colonne de chromatographie comprenant un élément choisi parmi le groupe constitué par les séquences nucléotidiques, les séquences d'acides aminés et/ou des fragments de celles-ci selon l'invention et tels que définis ci-dessus. Ledit dispositif de diagnostic peut également comprendre un ou plusieurs réactif pour la détection et/ou le dosage de séquences nucléotidiques et/ou polypeptidiques basées sur les méthodes choisies parmi le groupe constitué par l'hybridation in situ, l'hybridation et/ou la reconnaissance par anticorps marqués, en particulier la technologie ELISA (Enzymes Linked Immuno-Sorbent Assay) ou RIA (Radio Immuno Assay), la détection sur filtre, sur support solide, en solution, en sandwich, en gel, par hybridation dot blot, Northern blot, Southern blot, marquage isotopique ou non isotopique (en particulier l'immunofluorescence ou la biotinilisation), la technique d'amplification génétique, la technique de double immunodiffusion, la technique de contre-électrophorèse, la

celles-ci) dans la composition pharmaceutique peut varier selon de très larges gammes, uniquement limitées par la fréquence d'administration, la tolérance et le niveau d'acceptation de la composition selon l'invention par le patient.

Un dernier aspect de la présente invention concerne l'utilisation du dispositif de diagnostic selon l'invention pour le diagnostic de maladies et/ou de lésions physiologiques chez l'homme ou l'animal, en particulier pour le diagnostic de maladies et/ou de lésions pulmonaires.

La présente invention concerne également l'utilisation de la composition pharmaceutique selon l'invention pour la préparation d'un médicament destiné au traitement et/ou à la prévention de maladies et/ou de lésions physiologiques chez l'homme ou l'animal, en particulier de maladies et/ou de lésions pulmonaires.

La présente invention sera décrite en détails dans les exemples suivants, en référence aux figures annexées.

**Brève description des figures**

La figure 1 représente une analyse par Northern blot de la présence de l'ARN messager encodant le polypeptide selon l'invention dans différents types de tissus humains.

La figure 2 représente une analyse par Northern blot de la présence de l'ARN messager encodant le polypeptide selon l'invention au niveau du poumon d'un rat après administration de lipopolysaccharides (LPS) induisant une réaction inflammatoire du poumon.

La figure 3 représente une analyse par Northern blot de la présence de l'ARN messager encodant le polypeptide B18 selon l'invention au niveau du poumon d'un rat après une injection intra-péritonéale d'agents pneumotoxiques.

La figure 4 représente l'alignement de la séquence nucléotidique selon l'invention avec une séquence nucléotidique connue (U82615).

La figure 5 représente la séquence nucléotidique SEQ ID NO 1 selon l'invention.

**Exemple 1 : Homologie de la séquence SEQ ID NO 1 avec des séquences connues**

Le polypeptide B18 (figures 5 et 6) de l'invention a été aligné avec des séquences homologues de protéines connues présentes dans les banques de données (GenBank, EMBL, DDBJ, PDB) ainsi qu'avec des séquences EST présentes dans GenBank. Ces résultats sont repris dans le tableau 1 ci-dessous.

**Tableau 1 : Homologies entre la protéine B18 (162 acides aminés) et d'autres protéines**

| Nom | Code identification NCBI ID | Identité (% d'homologie) |
|---|---|---|
| Protéine de membrane (synechocystis sp.) | 1652859 | 33/60 (55%)<br>8/19 (42%)<br>9/23 (39%) |
| Lipomyces kononenkoae putative peroxisomal protein | 558080 | 32/75 (42%)<br>7/23 (30%)<br>7/18 (43%) |

| Nom | Code identification NCBI ID | Identité (% d'homologie) |
|---|---|---|
| Protéine hypothétique Haein HI0572 | 1723174 | 32/76 (42%)<br>10/26 (38%) |
| Non connu (Rhizobium sp.) | 1486441 | 31/61 (50%)<br>8/20 (40%) |
| Protéine A de la membrane peroxysomale (PMP20) | 130360 | 29/69 (42%)<br>8/14 (57%) |
| Protéine D de la membrane peroxysomale (PMP20) | 130361 | 30/82 (36%)<br>8/14 (57%) |
| Protéine peroxysomale PMP yeast putative | 1709682 | 12/33 (36%)<br>8/28 (28%)<br>7/11 (63%) |
| Protéine alkylhydroperoxyde réductase | 1591451 | 14/44 (38%)<br>8/28 (28%) |

Le tableau 2 reprend les pourcentages d'homologie entre le cDNA de la protéine B18 (805 nucléotides) avec d'autres séquences nucléotidiques.

5

Tableau 2

| Nom | Numéro d'accès | Identité |
|---|---|---|
| mRNA humain sous la régulation de cellules infectées par des adenovirus 5 | U82616 | 273/292 (93%) |
| | U82615 | 129/136 (94%)<br>99/108 (91%)<br>74/105 (70%) |

La figure 4 représente l'alignement de la séquence nucléotidique du B18 et de la séquence

nucléotidique U82615 du tableau 2.

Le listing ci-dessous reprend les séquences EST présentant une homologie avec le cDNA de la protéine B18 (805 nucléotides).

*Humain :*

AA130751, N42215, W38597, N91311, N68467, AA187737, N68916, W00593, R88950, AA181884, H20154, H66666

*Souris :*

AA220019, AA123351, AA087129, AA255021, AA249897, W71344

**Exemple 2**

Une analyse par Northern blot dans différents tissus humains de l'ARN messager encodant le polypeptide B18 humain selon l'invention et représentée sur la figure 1 annexée donne une révélation particulière au niveau du poumon.

Cette analyse a été obtenue à partir d'une trousse Multiple Tissues Northern Blot ® (Clontech), comprenant approximativement 2 µg d'une séquence poly-A et d'un ARN humain dans chacune des lignes hybridées avec une sonde B18 de 554 paires de bases fixée et reportée avec une sonde de ß-actine de 2 kb; toutes deux marquées à l'élément radioactif $^{32}P$.

L'analyse par Northern blot a été déterminée par Phosphorimaging Technology et les données relatives au mRNA du polypeptide B18 ont été normalisées par rapport au niveau de mRNA de la ß-actine.

Exemple 3

Une analyse par Northern blot du mRNA codant pour la protéine B18 du rat a été analysée au niveau du poumon du rat après 6, 48 et 72 heures suivant l'administration au rat de lipopolysaccharides (LPS) induisant une réaction inflammatoire au niveau du poumon.

Cette analyse a été obtenue par Nothern Blot au moyen de 15 µg de RNA total hybridé sur chaque bande avec une sonde de 225 paires de base encodant la protéine B18 du rat, fixée et reportée sur une sonde de 572 paires de base encodant la ß-actine du rat; les deux sondes étant marquées à l'élément radioactif $^{32}P$.

L'analyse par Northern blot a été quantifiée par Phosphorimaging Technology et les données relatives au mRNA encodant le polypeptide B18 ont été normalisées par rapport au niveau de mRNA de la ß-actine.

La figure 2 indique que l'on peut utiliser les séquences selon l'invention comme marqueurs d'une infection inflammatoire au niveau des poumons.

Exemple 4

Les Inventeurs ont fait une analyse par Northern blot de la présence de mRNA encodant le polypeptide B18 au niveau d'un poumon de rat après injection intra-péritonéale d'agents pneumotoxiques.

Ces agents sont le 4-ipoméanol,1-(3-fyryl)-4-hydroxypentanone (IPO), le méthylcyclopentadiényle manganèse tricarbonyle (MMT) et le α-naphtylthiourée (ANTU).

Ces agents sont connus pour induire au niveau des poumons des lésions aiguës des cellules de clara (IPO) ou des lésions aiguës au niveau des cellules alvéolaires

(MMT) ainsi que pour provoquer une augmentation de la perméabilité de la barrière sanguine au niveau des alvéoles (ANTU).

L'analyse par Northern blot a été effectuée au moyen de 15 µg de RNA total hybridé sur chaque bande avec une sonde de 225 paires de base encodant le polypeptide B18 du rat, fixée et reportée sur une sonde de 572 paires de base encodant la ß-actine du rat; les deux sondes étant marquées à l'élément radioactif $^{32}P$.

L'analyse par Northern blot a été quantifiée par la Phosphorimaging Technology et les données relatives au mRNA du polypeptide B18 ont été normalisées par rapport au niveau de mRNA de la ß-actine.

La figure 3 montre que les séquences nucléotidiques selon l'invention peuvent être utilisées comme marqueurs de lésions induites par l'injection d'agents pneumotoxiques connus ou non connus.

## REVENDICATIONS

1. Séquence d'acides nucléiques présentant plus de 70% d'homologie avec la séquence SEQ ID NO 1 ou son brin complémentaire.

2. Séquence d'acides nucléiques présentant plus de 85% d'homologie avec la séquence SEQ ID NO 1 ou son brin complémentaire.

3. Séquence d'acides nucléiques présentant plus de 95% d'homologie avec la séquence SEQ ID NO 1 ou son brin complémentaire.

4. Séquence d'acides nucléiques correspondant à la séquence SEQ ID NO 1, son brin complémentaire ou des portions de ceux-ci comprenant plus de 15 paires de base, susceptible d'identifier ou de reconstituer la séquence SEQ ID NO 1.

5. Séquence d'acides aminés présentant plus de 70% d'homologie avec la séquence SEQ ID NO 1.

6. Séquence d'acides aminés présentant plus de 85% d'homologie avec la séquence SEQ ID NO 1.

7. Séquence d'acides aminés présentant plus de 95% d'homologie avec la séquence SEQ ID NO 1.

8. Séquence d'acides aminés correspondant à la séquence SEQ ID NO 1 ou une portion de celle-ci.

9. Anticorps dirigé contre les séquences selon l'une quelconque des revendications précédentes.

10. Dispositif de diagnostic comprenant un élément choisi parmi le groupe constitué par les séquences d'acides nucléiques, les séquences d'acides aminés, des portions de celles-ci et/ou les anticorps selon l'une quelconque des revendications précédentes.

11. Dispositif selon la revendication 10, caractérisé en ce qu'il est une trousse de diagnostic ou

une colonne de chromatographie.

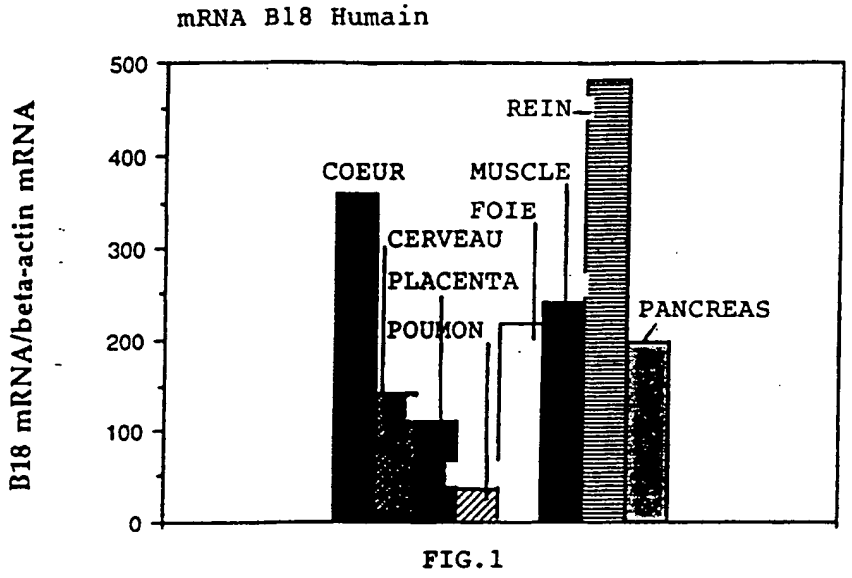


FIG.1

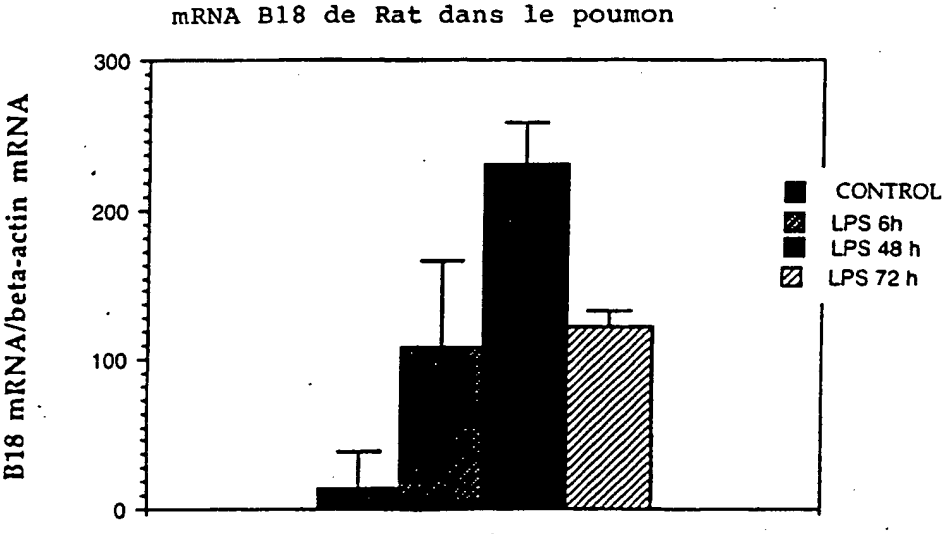


FIG.2

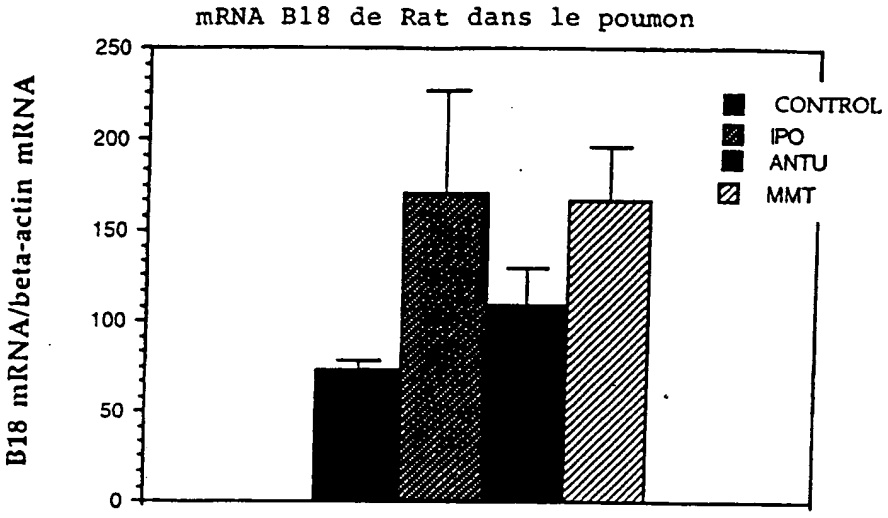
mRNA B18 de Rat dans le poumon
B18 mRNA/beta-actin mRNA
250
200
150
100
50
0
CONTROL
IPO
ANTU
MMT

FIG.3

```
U82615          TCAGTATCGGCGGAATTCGXXXTXXXXTCXAXXGGATTGGAATTGGCCTT
B18             ----------------------GCCAGG--AGGCGGAGTGGAAGTGGCCGT
                                        *** ***** ***** *

U82615          GGGGCGGGTTTGGGACTAGCTGGCGTGTGCGCCCTGAXACGCTCAGCGGG
B15             GGGGCGGGTATGGGACTAGCTGGCGTGTGCGCCCTGAGACGCTCAGCGGG
                ********* ************************** ***********

U82615          CTATATACTCGTCGGTGGGGCCGGCGGTCAGTCTGCGGCAGCGGCAGCAA
B19             CTATATACTCGTCGGTGGGGCCGGCGGTCAGTCTGCGGCAGCGGCAGCAA
                **************************************************

U82615          XACGGTGCAGTGAAGGAAAAXTGGGCGTCTGGCGGGGTCCGCAGTTTCAG
B18             GACGGTGCAGTGAAGGAGA-GTGGGCGTCTGGCGGGGTCCGCAGTTTCAG
                 **************** *  ****************************

U82615          CAAAGCCGCTGCAGCCATGGCCCCAATCAAGGTGGGAGATGCCATCCCAG
B18             CAGAGCCGCTGCAGCCATGGCCCCAATCAAGGTGGGAGATGCCATCCCAG
                ** **************************************************

U82615          CAXTGGAGGTGTTTTGAAGGGGAGCCAGGGAACAAGGTTGAACCTGGCAA
B18             CAGTGGAGGTGTTT-GAAGGGGAGCCAGGGAACAAGGT-GAACCTGGCAG
                ** ************ ************************ **********

U82615          AXCTGTTCAAXGGCAAAAAGGTTGTGCTGTTTGGAATTCCCXGGGGCCTC
B18             AGCTGTTCAAGGGCAAGAAGGGTGTGCTGTTTGGAGTTCC-TGGGGCCTT
                * ********* ***** **** ************** ****  ********

U82615          CACCCCTGAXTTTTCCCAAAAXCACCTTCCCAGGTTT--------------
B18             CACCCCTGGATGTTCCAAGACACACCTGCCAGGGTTTGTGGAGCAGGCTG
                ********  * **** * *  ***** **  *****

U82615          ---TTTTXAA---CAAGX-----------------------TTAA-
B18             AGGCTCTGAAGGCCAAGGGAGTCCAGGTGGTGGCCTGTCTGAGTGTTAAT
                    * * **   ****                          ****

U82615          ---GCXCCTA---------------------------AAGGC------CAX
B18             GATGCCTTTGTGACTGGCGAGTGGGGCCGAGCCCACAAGGCGGAAGGCAA
                   **   *                            *****     **

U82615          GA----ATTCCCG--------TXXXXGGXCCTTT----------------
B18             GGTTCGGCTCCTGGCTGATCCCACTGGGGCCTTTGGGAAGGAGACAGACT
                *       *** *              ** *****

U82615          -----TCAAAT----------------TTTTTAAAAACCX---TTTTAAX
B18             TATTACTAGATGATTCGCTGGTGTCCATCTTTGGGAATCGACGTCTCAAG
                      * **          .     * ***   ** *   * * **

U82615          XX----------------CAAAXTTG---------GGCCCCAAXCC----
B18             AGGTTCTCCATGGTGGTACAGGATGGCATAGTGAAGGCCCTGAATGTGGA
                              **   * *         *****  *

U82615          -CCAAAAGXCAAAAX-----------------------------------
B18             ACCAGATGGCACAGGCCTCACCTGCAGCCTGGCACCCAATATCATCTCAC
                 *** * * ** *

U82615          ------GA-----------AGGTT--TTCCCCCCCCCC------------
B19             AGCTCTGAGGCCCTGGGCCAGATTACTTCCTCCACCCCTCCCTATCTCAC
                      **           ** **  **** ** ****

U82615          -------------------------GCAA--------------------
B19             CTGCCCAGCCCTGTGCTGGGGCCCTGCAATTGGAATGTTGGCCAGATTTC
                                        ****

U82615          -------ACCCCCXXTGGXC-----------G
B18             TGCAATAAACACTTGTGGTTTGCGGAAAAAAA
                      * * *   ***
```

FIG. 4

Sequence B18 Protéine: pI (théorique): 6,96-7,3 MW: 16.899
aa: 162

```
                                                                    60
GCCAGGAGGCGGAGTGGAAGTGGCCGTGGGGCGGGTATGGGACTAGCTGGCGTGTGCGCC

                                                                   120
CTGAGACGCTCAGCGGGCTATATACTCGTCGGTGGGGCCGGCGGTCAGTCTGCGGCAGCG

                                                                   180
GCAGCAAGACGGTGCAGTGAAGGAGAGTGGGCGTCTGGCGGGGTCCGCAGTTTCAGCAGA

                                                                   240
GCCGCTGCAGCCATGGCCCCAATCAAGGTGGGAGATGCCATCCCAGCAGTGGAGGTGTTT
              M  A  P  I  K  V  G  D  A  I  P  A  V  E  V  F

                                                                   300
GAAGGGGAGCCAGGGAACAAGGTGAACCTGGCAGAGCTGTTCAAGGGCAAGAAGGGTGTG
  E  G  E  P  G  N  K  V  N  L  A  E  L  F  K  G  K  K  G  V

                                                                   360
CTGTTTGGAGTTCCTGGGGCCTTCACCCCTGGATGTTCCAAGACACACCTGCCAGGGTTT
  L  F  G  V  P  G  A  F  T  P  G  C  S  K  T  H  L  P  G  F

                                                                   420
GTGGAGCAGGCTGAGGCTCTGAAGGCCAAGGGAGTCCAGGTGGTGGCCTGTCTGAGTGTT
  V  E  Q  A  E  A  L  K  A  K  G  V  Q  V  V  A  C  L  S  V

                                                                   480
AATGATGCCTTTGTGACTGGCGAGTGGGGCCGAGCCCACAAGGCGGAAGGCAAGGTTCGG
  N  D  A  F  V  T  G  E  W  G  R  A  H  K  A  E  G  K  V  R

                                                                   540
CTCCTGGCTGATCCCACTGGGGCCTTTGGGAAGGAGACAGACTTATTACTAGATGATTCG
  L  L  A  D  P  T  G  A  F  G  K  E  T  D  L  L  L  D  D  S

                                                                   600
CTGGTGTCCATCTTTGGGAATCGACGTCTCAAGAGGTTCTCCATGGTGGTACAGGATGGC
  L  V  S  I  F  G  N  R  R  L  K  R  F  S  M  V  V  Q  D  G

                                                                   660
ATAGTGAAGGCCCTGAATGTGGAACCAGATGGCACAGGCCTCACCTGCAGCCTGGCACCC
  I  V  K  A  L  N  V  E  P  D  G  T  G  L  T  C  S  L  A  P

                                                                   720
AATATCATCTCACAGCTCTGAGGCCCTGGGCCAGATTACTTCCTCCACCCCTCCCTATCT
  N  I  I  S  Q  L  *

                                                                   780
CACCTGCCCAGCCCTGTGCTGGGGCCCTGCAATTGGAATGTTGGCCAGATTTCTGCAATA

                                                                   840
AACACTTGTGGTTTGCGGAAAAAAA
```

FIG.5